## REMARKS/ARGUMENTS

## THE INVENTION

This invention is a novel process for enriching for genes and regulatory sequences within a eukaryote genome by taking advantage of the differences in Cot values between the desired genes and regulatory sequences and undesired repetitive DNA.

## STATUS OF THE CLAIMS

Claims 46-57 are pending. All claims are objected to or rejected under §112. Claims 46, 47, 48, 50, 55 and 57 are amended. There is no new matter added. The amendments to claim 46 correct grammatical errors and provide more precise antecedent basis. Claim 47 was amended to correct a grammatical error. Claims 48 and 50 are amended to more precisely correspond to the language used in claim 46. Claims 55 and 57 are amended to place the word genome after the type of organism being studied.

## OBJECTIONS

Oath:

The Examiner has stated that the Rule 63 declaration is defective because it failed to list the newly added inventor, Pablo D. Rabinowicz. A supplemental declaration signed by all three named inventors was filed on June 8, 2006. A copy of the declaration is attached to this Response as **Exhibit 1**.

Drawings:

Figures 3 and 9 were stated as not observable. Applicants have had Figure 3 redone and ask for clarification on Figure 9. It is not clear what portion of Figure 9 requires attention. Observable copies of both are provided herewith.

Specification:

The Examiner asked that the *incorporation by reference* language on page one be deleted, and applicants have amended the paragraph as suggested by the Examiner.

The hyperlinks on pages 18 and 19 were objected to and have been deleted by amendment.

The description of Figure 4 has been amended to delete reference to subparts A and B.

Claim Objections:

The Examiner has objected to claim 46 asking that a spelling error be corrected. Claim 47 was objected because of an improper article. Claims 55 and 57 were objected because of a grammatical concern over the placement of the word, "genome". Applicants have amended the claims as requested by the Examiner.

## REJECTIONS

35 U.S.C. §112 2nd ¶

Claims 46, 47 and 49-57 were rejected as indefinite.

Claim 46 was rejected as indefinite for failing to recite specific dimensions for $C_0t$. $C_0t$ is a standard scientific value. It refers to a number value defined by moles of nucleotides per liter times multiplied by seconds. It is a term of art, and by convention, those of skill often do not set forth its dimensions. Attached to this response is Exhibit 2 (a-e) comprising two definitions of $C_0t$ and three references using $C_0t$. The accepted definition is as the examiner states: *c*oncentration x *t*ime. From a text book entitled Genes IV by Lewin, the concentration is given in moles of nucleotides per liter, and the time is in seconds (definition from Everything Bio). The PNAS paper of Leibovitch set forth $C_0t$ dimensions on page 3721, 2nd column, as nucleotide concentration (mol/liter) x time (sec). Evidence of the convention of omitting dimensions for $C_0t$ value is found in Leibovitch *et al.* on page 3722, first column (reciting $C_0t$ value of less than 100...); in Fouquet *et al.*, at page 35 (reciting $C_0t$ value of 15); and Hubbell *et al.*, (abstract) and at page 2445 ( reciting $C_0t$ value of $7.2x10^{-2}$).

Having explained that recitation of $C_0t$ value is often dimensionless and that it has by convention only one meaning, applicants have not amended the pending claims. However, if the Examiner believes the term requires recitation of dimensions (mol of nucleotides x sec x liters$^{-1}$) applicants will amend the claims as suggested.

Claim 46 was further rejected for recitation of "the non-repetitive population." As amended, the claim now recites "single stranded population of non-repetitive fragments enriched for genes." The amended language corresponds precisely to the language used earlier in claim 46 and thus addresses the antecedent basis concern raised by the Examiner.

Claim 48 was rejected as lacking antecedent basis for recitation of "the genomic DNA." As amended, claim 48 now recites "eukaryotic genome." The amended language corresponds to the language of claim 46, and applicants submit that this amendment fully addresses the antecedent basis issue.

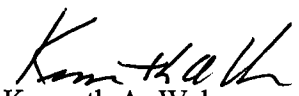
Finally, claim 50 was rejected as lacking antecedent basis for recitation of "genomic DNA fragments." As amended, claim 50 now recites "fragments." The amended language corresponds to the language of claim 46, and applicants submit that this amendment fully addresses the antecedent basis issue.

## CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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KAW:kaw/jhd
61003521 v1

Attorney Docket No.: 021031-000113US
Client Ref. No.:

PTO/SB/01A (09-04)

# DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | GENETICALLY FILTERED SHOTGUN SEQUENCING OF COMPLEX EUKARYOTIC GENOMES |
|---|---|

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 10/656,482, filed on September 5, 2003,

☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: W. Richard McCombie
Signature: W. Richard McCombie Citizen of: United States

Inventor two: Robert A. Martienssen
Signature: R. A. Martienssen Citizen of: United Kingdom

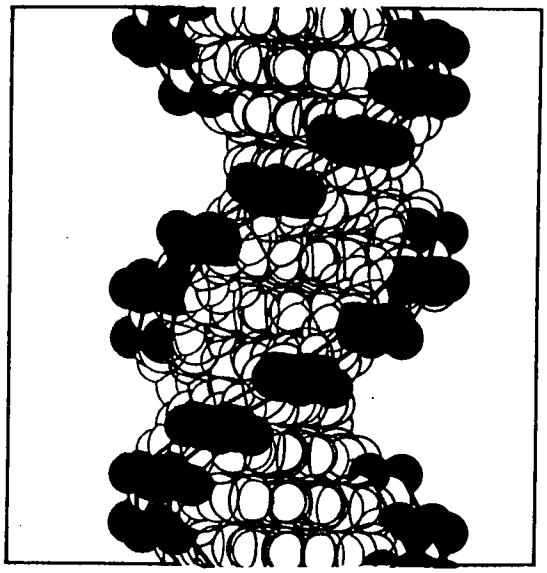
Inventor three: Pablo D. Rabinowicz
Signature: Citizen of: Argentina

Inventor four:
Signature: Citizen of:

☐ Additional inventors or a legal representative are being named on _____ additional form(s) attached hereto.

60356565 v1

# EXHIBIT 1



# GENES IV

## BENJAMIN LEWIN

1990
Oxford University Press
Oxford New York Tokyo Melbourne
Cell Press, Cambridge

**EXHIBIT 2A**

be counted in tens of thousands rather than in hundreds of thousands.

Some direct evidence is gained by counting the number of genes via estimates of the number of different mRNAs (see later). While this cannot be done for every cell type (to generate a sum total for the organism), it seems that the number of genes expressed in a given cell type is ~10,000. Most of these genes are common to most or all cells of the organism. So this value is probably within a factor of (say) 2–4 of the total expressed gene number. Given some uncertainties about estimating the numbers of genes present in multiple copies, we might say that the mammalian genome could contain 30,000–40,000 gene functions.

When we characterize genes corresponding to individual functions, we often find additional copies representing unsuspected variants of the gene. The extension from individual genes to families of related genes certainly increases the number of genes in the genome. Does it increase it 10× to account for all the DNA? We do not know the answer.

A less direct line of evidence is provided by attempts to estimate the number of essential genes by identifying all the loci that can be mutated. Much of this work has been performed with *D. melanogaster,* where we can infer that the total essential gene number is likely to be ~5000. With a reasonable estimate for the size of the insect gene as 2000 bp, this corresponds to a total length of $10^7$ bp, just 10 times less than the amount of available DNA. Similarly, with *C. elegans* on average there is 1 essential gene for every 30,000 bp, a distance probably 10× greater than the size of the average gene. It seems likely that there are quite sizeable regions between genes, and we have yet to define their nature.

Of course, the genes identified by mutation are those in which damage has visible or lethal effects. Perhaps only some genes fall into this class. This would imply that at least a large proportion, possibly even the majority, of genes are concerned with specifying proteins that are not essential for the survival of the organism (at least in the sense that mutational damage does not cause any detectable effect). Molecular analysis of chromosomal regions of *D. melanogaster* suggests that the total number of genes could be 3–4× the number of essential genes (see Chapter 20).

Analysis of the genome of the yeast *S. cerevisiae* has produced a surprising result. The genome is relatively small, and a large fraction (>50%) is transcribed, compared with higher eukaryotes. When insertions were introduced at random into the genome, only 12% were lethal, and another 14% impeded growth. This result implies that ~75% of the yeast genome is dispensable at least under the conditions of the experiment.

Assume that genes account for half of the yeast genome, and that the disruptions are equally distributed between genic and nongenic regions. If all the insertions that are deleterious reside in the half of the genome that is occupied by genes, then half of the genes may be nonessential.

So we are left with several key questions. What proportion of the DNA of the genome actually is concerned with representing proteins in the sense that it lies in a gene, either in the coding region itself or in an intervening or transcribed flanking sequence? Of the number of genes, how many are essential and how many dispensable? What is the function (if any) of DNA that does not reside in genes? What effect does a large change in total size have on the operation of the genome, as in the case of the related amphibians?

## Reassociation Kinetics Depend on Sequence Complexity

Reassociation between complementary sequences of DNA occurs by base pairing, in a reversal of the process of denaturation by which they were separated (see Figure 5.2). The technique can be extended to isolate individual DNA or RNA sequences by their ability to hybridize with a particular probe (see Chapter 23). The kinetics of the reassociation reaction reflect the variety of sequences that are present; so the reaction can be used to quantitate genes and their RNA products. When performed in solution, such reactions are described as **liquid hybridization.**

Renaturation of DNA depends on random collision of the complementary strands, and follows second-order kinetics. The rate of reaction is governed by the equation

$$\frac{dC}{dt} = -kC^2 \qquad (1)$$

where C is the concentration of DNA that is single-stranded at time $t$, and $k$ is a reassociation rate constant.

By integrating this equation between the limits of the initial concentration of DNA, $C_0$ at time $t = 0$, and the concentration C that remains single-stranded after time $t$, we can describe the progress of the reaction as

$$\frac{C}{C_0} = \frac{1}{1 + k.C_0t} \qquad (2)$$

Thus when the reaction is half complete, at time $t_{1/2}$,

$$\frac{C}{C_0} = \frac{1}{2} = \frac{1}{1 + k.C_0t_{1/2}} \qquad (3)$$

so that

$$C_0t_{1/2} = \frac{1}{k} \qquad (4)$$

Equation 2 shows that the parameter controlling the reassociation reaction is the product of DNA concentration ($C_0$) and time of incubation ($t$), usually described simply as the **Cot.** The value required for half-reassociation ($C_0t_{1/2}$) is called the **$Cot_{1/2}$.** Since the $Cot_{1/2}$ is the product of the concentration and time required to proceed halfway, a greater $Cot_{1/2}$ implies a slower reaction.

The reassociation of DNA usually is followed in the

form of a **Cot curve,** which plots the fraction of DNA that has reassociated ($1 - C/C_0$) against the log of the Cot. **Figure 24.2** gives Cot curves for several genomes. The form of each curve is similar, with renaturation occurring over an ~100-fold range of Cot values between the points of 10% reaction and 90% reaction. But the Cot required in each case is very different. It is described by the $Cot_{1/2}$.

The $Cot_{1/2}$ is directly related to the amount of DNA in the genome. This reflects a situation in which, as the genome becomes more complex, there are fewer copies of any particular sequence within a given mass of DNA. For example, if the $C_0$ of DNA is 12 pg, it will contain 3000 copies of each sequence in a bacterial genome whose size is 0.004 pg, but will contain only 4 copies of each sequence present in a eukaryotic genome of size 3 pg. Thus the same *absolute* concentration of DNA measured in moles of nucleotides per liter (the $C_0$) will provide a concentration of each eukaryotic sequence that is 750× (3000/4) lower than that of each bacterial sequence.

Since the rate of reassociation depends on the concentration of complementary sequences, for the eukaryotic sequences to be present at the same *relative* concentration as the bacterial sequences, it is necessary to have 750× more DNA (or to incubate the same amount of DNA for 750 times longer). Thus the $Cot_{1/2}$ of the eukaryotic reaction is 750× the $Cot_{1/2}$ of the bacterial reaction.

The $Cot_{1/2}$ of a reaction therefore indicates the *total length of different sequences* that are present. This is described as the **complexity,** usually given in base pairs.

The renaturation of the DNA of any genome (or part of a genome) should display a $Cot_{1/2}$ that is proportional to its complexity. Thus the complexity of any DNA can be determined by comparing its $Cot_{1/2}$ with that of a standard DNA of known complexity. Usually *E. coli* DNA is used as a standard. Its complexity is taken to be identical with the length of the genome (implying that every sequence in the *E. coli* genome of $4.2 \times 10^6$ bp is unique). So we can write

$$\frac{Cot_{1/2}\text{ (DNA of any genome)}}{Cot_{1/2}\text{ (E. coli DNA)}} = \frac{\text{Complexity of any genome}}{4.2 \times 10^6 \text{ bp}} \quad (5)$$

## Eukaryotic Genomes Contain Several Sequence Components

When the DNA of a eukaryotic genome is characterized by reassociation kinetics, usually the reaction occurs over a range of Cot values spanning up to eight orders of magnitude. This is much broader than the 100-fold range expected from equation 2 and shown for the examples of **Figure 24.2**. The reason is that the equation applies to a single **kinetically pure** reassociating component. *A genome actually includes several such components, each reassociating with its own characteristic kinetics.*

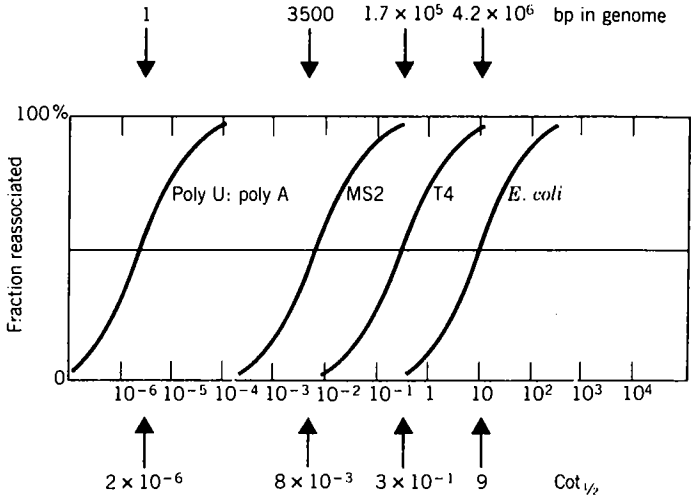
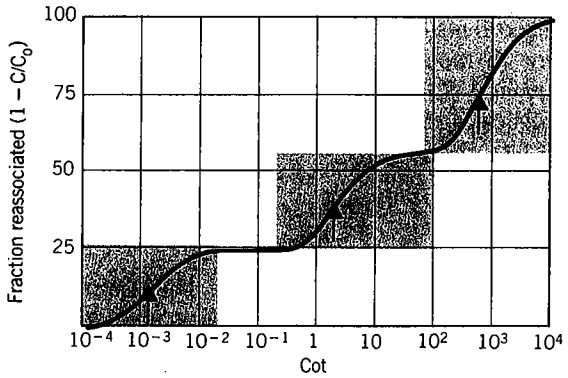



**Figure 24.2**
**Rate of reassociation is inversely proportional to the length of the reassociating DNA.**

Equation 4 shows that the reassociation of any particular DNA can be described by the $C_0t_{1/2}$ (given in nucleotide-moles × sec/liter) or in the form of its reciprocal rate constant $k$ (in units of liter-nucleotide-moles$^{-1}$ -sec$^{-1}$).

**Figure 24.3** shows the reassociation of a (hypothetical) eukaryotic genome, starting at a Cot of $10^{-4}$ and terminating at a Cot of $10^4$. The reaction falls into three distinct

**Figure 24.3**
**The reassociation kinetics of eukaryotic DNA show three types of component (indicated by the shaded areas).**




| | Fast component | Intermediate component | Slow component |
|---|---|---|---|
| Percent of genome | 25 | 30 | 45 |
| $Cot_{1/2}$ | 0.0013 | 1.9 | 630 |
| Complexity, bp | 340 | $6.0 \times 10^5$ | $3.0 \times 10^8$ |
| Repetition frequency | 500,000 | 350 | 1 |

Definition of cot value :

(cot1/2); The product of Co (the original concentration of denatured DNA) and t (time in seconds) useful index of DNA renaturation. Cot1/2 is the value when 50% renaturation has occurred which used to estimate the length of unique DNA in a sample.





**EXHIBIT 2B**

*Proc. Natl. Acad. Sci USA*
Vol. 74, No. 9, pp. 3720–3724, September 1977
Biochemistry

# Single-stranded DNA from oncornavirus-infected cells enriched in virus-specific DNA sequences

(hydroxyapatite chromatography/avian myeloblastosis virus/molecular hybridization/provirus DNA transcription)

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*Communicated by Charlotte Friend, June 13, 1977*

ABSTRACT **We previously found that a minor fraction of single-stranded DNA (ss-DNA) isolated from native nuclear DNA of normal chicken embryonic cells and cells of other species hybridized with bulk nuclear DNA or cellular RNA in great excess. At least one-third of ss-DNA belonging to the nonrepetitious part of the cell genome could be hybridized to homologous RNAs. In the present work, similar results were obtained with ss-DNA from cells of chickens infected by avian myeloblastosis virus (AMV). To investigate whether this enrichment of ss-DNA in transcribed DNA sequences involves provirus DNA, radioactive AMV RNA and cDNA copies of AMV RNA were used. Most of the 70S AMV RNA hybridized much faster to ss-DNA from productively infected leukemic cells than to bulk DNA. cDNA, either double-stranded or single-stranded, made in the presence of actinomycin D hybridized to total nuclear DNA with similar kinetics. In contrast, about half of the double-stranded cDNA molecules hybridized 40–50 times faster to ss-DNA than to total DNA, indicating that only one of the provirus DNA strands seems to be present in ss-DNA. This was confirmed by the fact that relatively insignificant amounts of the ss-cDNA molecules made in the presence of actinomycin D could be annealed to ss-DNA as compared with bulk DNA. These results indicate that actively transcribed DNA sequences can be selectively distributed in the ss-DNA fraction, probably because of single strand breaks in the vicinity of transcription sites.**

The provirus theory of Temin (1), which postulates that the replication of RNA tumor viruses proceeds via DNA copies of viral RNA integrated in the host genome, has been substantiated by the discovery of successful transfection mediated by reverse transcriptase (2, 3), through an intermediate of viral DNA isolated from oncornavirus-transformed cells (4–6) and molecular hybridization between the viral genome and cell nucleic acids (7, 8). In productively infected cells, the integrated provirus DNA appears to be more actively transcribed than most of the cellular genes (9).

We have isolated, from the nDNA of various species, a minor fraction of single-stranded DNA (ss-DNA) and demonstrated that about one-third of the ss-DNA from cultured normal embryonic chicken cells (10) or human RD cells (11) can hybridize to homologous mRNAs. These and other characteristics suggested that ss-DNA arises from duplex molecules destabilized during RNA synthesis and probably split in the course of DNA isolation. If this is true, then ss-DNA of oncornavirus productive cells must be enriched in provirus DNA sequences as compared with total DNA of the same cells and ss-DNA of nonproductive cells. The present work demonstrates that this is indeed the case and provides evidence for preferential single strand breaks in the vicinity of transcribed DNA sites.

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## MATERIALS AND METHODS

**Cells and Labeling Procedures.** Leukemic cells collected at the terminal stage of leukemia from the blood of 3- to 4-week-old chickens infected with avian myeloblastosis virus (AMV), BAI strain A, were washed three times with 199 medium. Approximately $2.5 \times 10^9$ cells in 50 ml of 199 enriched medium (12) were transferred to a 500-ml bottle, and [$^3$H]-thymidine (50 $\mu$Ci/ml; specific activity, 46 Ci/mmol) or [$^3$H]uridine (50 $\mu$Ci/ml; specific activity, 30 Ci/mmol) was added. The cells were incubated for 24 hr at 37° with slow rotation. The pH of the medium was maintained at 7.4 during the course of incubation. The primary cell cultures prepared from 10-day-old chick embryos were obtained as described (13).

**Preparation of DNA from Leukemic or Normal Cells.** Cell nuclei were purified by using nonionic detergent (14), resuspended in 0.01 M Tris-HCl, pH 8.3/0.01 M EDTA, and lysed by incubation for 30 min at 37° in the presence of 0.1% sodium dodecyl sulfate and Pronase (100 $\mu$g/ml). The concentration of sodium dodecyl sulfate was increased to 1% and the nuclei were reincubated for 30 min at 37°. After addition of sodium perchlorate to 1.0 M, the DNA was extracted three times with chloroform/isoamyl alcohol, 99:1 (vol/vol), precipitated with ethanol, and redissolved in 0.1× standard citrate–saline solution (SSC). Pancreatic RNase (20 $\mu$g/ml) and RNase $T_1$ (20 units/ml) were added and the solution was incubated for 30 min at 37°. After reextraction the DNA was finally precipitated with ethanol and redissolved in and dialyzed against 0.014 M NaCl/0.01 M Tris, pH 8.3/0.002 M EDTA.

**Isolation of ss-DNA.** The modification of the method for hydroxyapatite chromatography used in these studies has been described (15). Briefly, elution was carried out at 56° with pH 7.85 phosphate buffer and, instead of column chromatography, a batch procedure was used. Under these conditions the separation of the ss- and double-stranded (ds)-DNA is greatly improved. The ss-DNA and ds-DNA fractions were dialyzed against 0.1× SSC and sonicated with a Branson sonifier $B_{12}$ for 20 sec in an ice bath at position 6 to obtain DNA fragments of 5–7 S as measured by alkaline sucrose gradient centrifugation.

**Synthesis of AMV [$^3$H]cDNA.** The standard endogenous RNA-dependent DNA polymerase reaction of Leis and Hurwitz (16, 17), which provides DNA copies (cDNA) representing more than 60% of the AMV genome, was used with and without addition of actinomycin D (AMD), which is known to inhibit the DNA-dependent reaction (18). The reaction mixture con-

Abbreviations: ss-DNA, single-stranded DNA; AMV, avian myeloblastosis virus; SSC, standard citrate–saline solution (0.15 M NaCl/0.015 M Na citrate, pH 7); ds-DNA, double-stranded DNA; AMD, actinomycin D; $C_0t$, nucleotide concentration (mol/liter) × time (sec).



EXHIBIT 2C

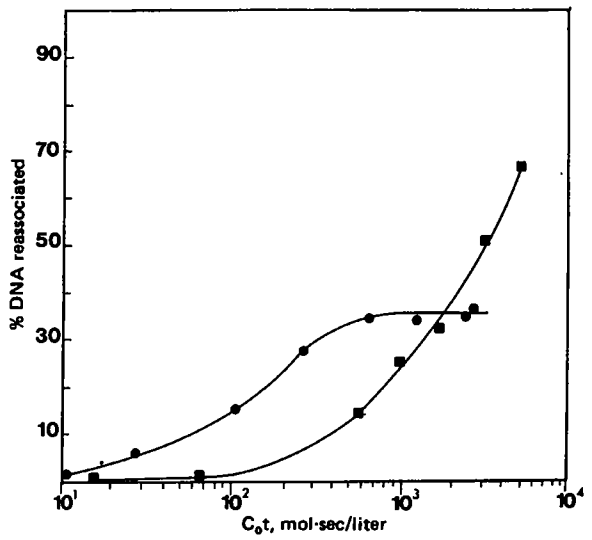


FIG. 1. Reassociation kinetics of ss-DNA from chicken leukemic cells. [$^3$H]Thymidine-labeled ss-DNA (●) was incubated at 66° in 2× SSC containing 0.1% sodium dodecyl sulfate, for different times. The amounts reassociated were determined by measuring the resistance to $S_1$ nuclease as indicated in *Materials and Methods*. As control (■), nonrepeated DNA sequences, selected by hydroxyapatite chromatography of bulk nuclear DNA renatured at $C_0t$=300 mol·sec/liter after alkaline denaturation, were reassociated under the same conditions.

tained (per ml): 55 mM Tris·HCl, pH 8.3; 40 mM KCl; 12.5 mM $MgCl_2$; 30 mM dithiothreitol; 0.1% Nonidet P-40; 10 μM dGTP; 10 μM dTTP; 400 μCi of [$^3$H]dCTP (specific activity, 25.5 Ci/mmol); 100 μg of AMD, and about 2 mg of AMV protein. After incubation for 4 hr at 37°, dATP, dCTP, dGTP, and dTTP (each 0.025 μm) were added and the mixture was incubated for 16 hr at 37°. In the absence of AMD, the same reaction mixture was used except that it contained 0.80 μM dGTP, 0.80 μM dATP, 37.5 μCi of [$^3$H]dCTP (specific activity, 25 Ci/mmol) and 60 μCi of [$^3$H]dTTP (specific activity, 16.6 Ci/mmol) per ml and was incubated for 2 hr at 41°.

**Purification of [$^3$H]cDNA.** After incubation, the reaction mixtures were adjusted to 0.25% in sodium dodecyl sulfate and 100 μg/ml in Pronase and were incubated at 37° for 30 min. The products were extracted twice with an equal volume of chloroform/isoamyl alcohol after addition of 150 μg of yeast RNA as carrier, precipitated at −20° in the presence of 0.2 M NaCl with 2 volumes of ethanol, centrifuged at 20,000 × *g* for 15 min, resuspended in 0.01 M phosphate buffer, pH 7.85, and chromatographed on hydroxyapatite. The greatest part of cDNA (specific activity, $1 \times 10^7$ cpm/μg) synthesized in the presence of AMD eluted as ss-DNA and only this part was used for hybridization experiments. The main cDNA fraction synthesized in the absence of AMD eluted as ds-DNA and only this fraction was used for hybridization. Each cDNA fraction was dialyzed against 0.1× SSC, adjusted to 0.1 M NaOH, incubated for 18 hr at 37°, neutralized by addition of 1 volume of cold 2 M $NaH_2PO_4$, dialyzed against 0.1× SSC, and precipitated with ethanol.

**Hybridization of AMV cDNA and AMV RNA.** Aliquots of AMV [$^3$H]cDNA were mixed with unlabeled cell DNA as indicated in Figs. 2 and 3. After heating for 10 min at 100° and fast cooling, hybridization was carried out by incubation at 66° in 2× SSC containing 0.1% sodium dodecyl sulfate, for different time periods. The extent of hybridization was determined by digestion with $S_1$ nuclease. Aliquots (5 or 10 μl) were diluted to a final volume of 250 μl in $S_1$ buffer (0.03 M Na acetate, pH 4.5/0.0018 M $ZnCl_2$/0.15 M NaCl) plus 15 μg of denatured calf thymus DNA with or without $S_1$ nuclease in excess. After incubation for 30 min at 40°, each sample was precipitated with trichloroacetic acid and filtered (Whatman glass-fiber, GF/B), and the filter was assayed for radioactivity in a liquid scintillation spectrometer. Results of hybridization were plotted as the percentage of $S_1$-resistant [$^3$H]cDNA versus the $C_0t$ value [nucleotide concentration (mol/liter) × time (sec)]. Hybridization of 70S AMV [$^3$H]RNA prepared as described (8, 12) was performed under similar conditions, except that RNases were utilized instead of $S_1$ nuclease and all solutions were pretreated with 1% diethylpyrocarbonate and heated for 10 min at 80°.

## RESULTS

**Isolation and Characterization of ss-DNA.** With the improved method of hydroxyapatite chromatography, about 2% of the native nuclear DNA from leukemic chicken cells was isolated as ss-DNA. Similar proportions of ss-DNA had been previously found in other cells including chicken embryonic cells (11, 15, 19, 20). Whatever its origin, the ss-DNA could be entirely degraded by DNase or $S_1$ nuclease and was resistant to RNase or alkaline treatment. When centrifuged in a CsCl density gradient, its mean buoyant density was the same as that of denatured bulk DNA. The mean sedimentation coefficient of ss-DNA, run through alkaline sucrose gradient, was 9–10 S, corresponding to 1–1.2 kilobases, whereas that of bulk DNA was about 18 S, corresponding to 6 kilobases (21). The molecular complexity of ss-DNA was analyzed by comparing its self-reassociation kinetics with those of the nonrepetitious portion of bulk DNA. As shown in Fig. 1, 30–32% of ss-DNA consisted of complementary chains reassociated at a mean $C_0t$ value of approximately 100 mol·sec/liter. The rest of the ss-DNA remained single stranded even after reaching $C_0t$ values of 3000–4000 mol·sec/liter, sufficient to allow renaturation of more than half of the nonrepeated sequences of bulk DNA. Similar results, obtained with three different preparations of DNA, indicated that the main part of ss-DNA seems to consist of fragments from only one of the DNA double strands. The complementary sequences of these non-self-reassociable fragments do exist in the cell genome because, like ss-DNA from other cells (10, 11), the ss-DNA from AMV-producing cells became hybridized to bulk nuclear DNA with the kinetics of nonrepeated DNA sequences. In addition, up to 35–36% of the ss-DNA from leukemic cells could be annealed with polysomal RNA as compared with 6–7% for bulk DNA [results not shown were similar to those obtained with ss-DNA from other cells (10, 11)].

Table 1. Characterization of cDNA probes

| Hydrolysis | AMV cDNA synthesized, % of nonhydrolyzed | |
|---|---|---|
| | Without AMD | With AMD |
| None | 100 | 100 |
| Alkali | 94.5 | 100 |
| DNase | 2.9 | 3.7 |
| $S_1$ nuclease | 61.8 | 33.5 |
| Denatured and $S_1$ | 6.4 | 2.5 |

**Characterization of AMV [$^3$H]cDNA.** The radioactive products of the endogenous DNA polymerase reactions were degraded by DNase but were resistant to RNases and alkali. The results of $S_1$ nuclease digestion (Table 1), confirmed by hy-

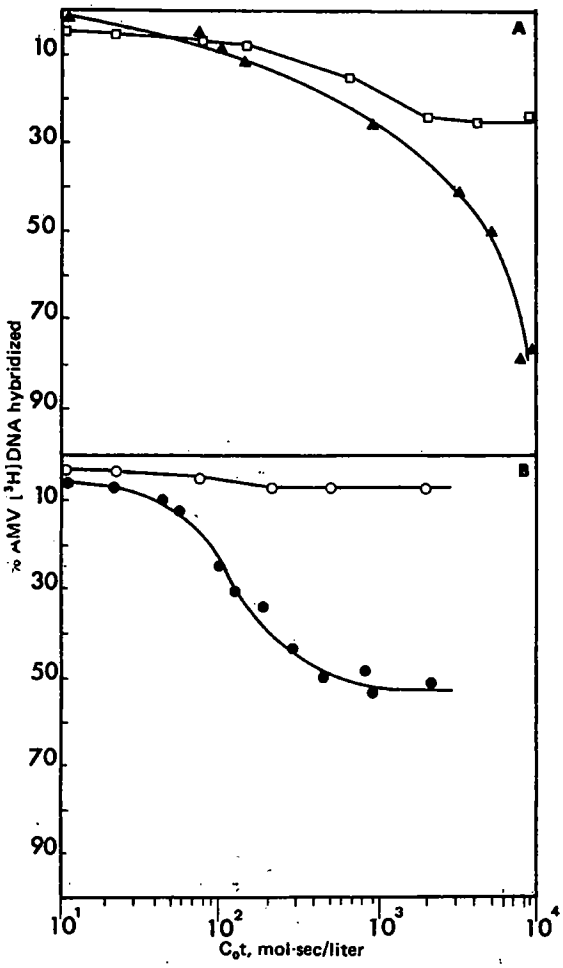


FIG. 2. Hybridization kinetics of AMV ds-[³H]cDNA (synthesized in absence of AMD) with cell DNA. (*A*) AMV cDNA hybridized to total nuclear DNA of uninfected cells (□) or of productively infected leukemic cells (▲) at a cell DNA-to-cDNA ratio of $7 \times 10^5$. (*B*) AMV cDNA hybridized to ss-DNA of the same uninfected (○) or productively infected (●) cells. The amounts hybridized were determined by measuring the resistance of [³H]cDNA to $S_1$ nuclease digestion.

droxyapatite chromatography, showed that the major part of the AMV cDNA synthesized in the presence of AMD consisted of ss-DNA chains and, in the absence of AMD, of ds-chains. No more than 40–45% of the latter cDNA probe hybridized to AMV 70S RNA in excess, as compared with 80–85% for the former. The fact that cDNA synthesized in the presence of AMD was not entirely digested by $S_1$ nuclease or totally hybridized to viral RNA is in agreement with the recent report (22) that AMD does not obligatorily inhibit all of the DNA-directed DNA synthesis.

**Hybridization of AMV cDNA and AMV RNA with Cellular DNA.** About 50% of the AMV ds-cDNA (made in the absence of AMD) hybridized to ss-DNA of productively infected leukemic cells at a mean $C_0t$ value of less than 100—that is to say, 40–50 times faster than to total DNA of the same cells (Fig. 2). No more than 25% of the same cDNA probe could be reassociated with total DNA from noninfected chicken cells and much less with ss-DNA. This agrees with recent data on the extent of homology between the provirus DNA sequences of endogenous and exogenous avian oncornaviruses (23). In three

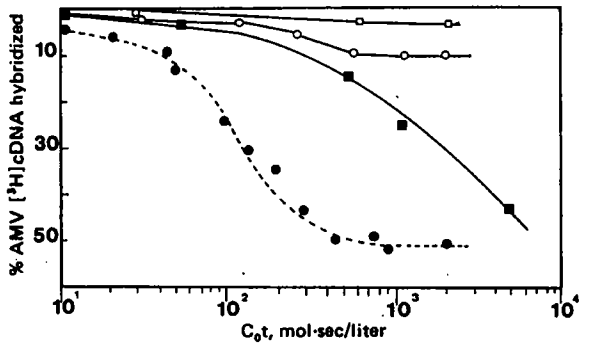


FIG. 3. Hybridization kinetics of AMVss-[³H]cDNA (synthesized in the presence of AMD) with cell DNA. AMV cDNA was hybridized to ss-DNA of noninfected cells (□) or total DNA (■) and ss-DNA (○) of productively infected chicken cells. Experimental conditions were the same as in the legend of Fig. 2. Hybridization of AMV ds-cDNA (synthesized in the absence of AMD) with ss-DNA of chicken leukemic cells (●) is shown to facilitate comparison.

different experiments, half of the AMV ds-cDNA molecules hybridized much faster to ss-DNA than to total DNA of AMV-producing cells but little more than half, even after reaching $C_0t$ values at which 80–85% of the same cDNA probe hybridized to total DNA. As illustrated by Fig. 3, at all $C_0t$ values, a much smaller proportion of the ss-cDNA copies synthesized in the presence of AMD hybridized to ss-DNA, compared with total DNA from leukemic cells, and insignificant amounts hybridized to ss-DNA from normal cells.

The double-stranded structure of cDNA-ssDNA hybrids was verified by hydroxyapatite chromatography and thermal fusion. The melting curve of these hybrids was almost as sharp as that of native cell DNA but their melting temperature was 5° lower. This difference was probably due to the small size of cDNA (4–5 S in sucrose gradient).

The selective enrichment of ss-DNA in transcriptable provirus sequences was confirmed by RNA-DNA annealing experiments (Fig. 4). Even for the low DNA/RNA ratio (1:6000), the major part of the purified 70S AMV RNA hybridized to ss-DNA from productively infected cells (with a $C_0t_{1/2}$ value of about 500) and relatively negligible amounts hybridized to total nuclear DNA or purified ds-DNA under the same conditions.

## DISCUSSION

The occurrence in eukaryotic cells of ss-DNA sequences (24–32), generally considered as DNA replication intermediates (25–29), products of alteration caused by cell aging (30, 31), or artifacts of the DNA extraction procedures (32), have been reported. We recently obtained evidence that suggested other mechanisms might be involved. The major part of ss-DNA could not consist of replication intermediates because its lifespan was similar to that of bulk DNA (10, 33). Another possibility that had to be considered is that ss-DNA is related to RNA synthesis. In effect, different transcription mechanisms have been proposed that imply unwinding of the DNA helix (34–37). Studying transcription of a phage DNA in a cell-free system, Bick *et al.* (38) used formamide, at a concentration below that required for partial denaturation of DNA, to visualize opened regions that involve 700–1500 DNA base pairs in the vicinity of the growing RNA chains. Groner *et al.* (39) found that the greatest part of the RNA synthesized in chromatin isolated from chicken leukemic cells was in the form of RNA-DNA hybrids. Our previous data provided evidence for DNA strand separa-

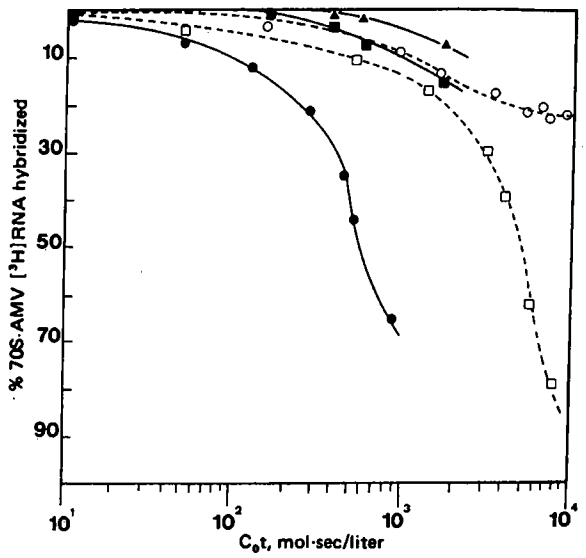


FIG. 4. Hybridization kinetics of purified 70S AMV [³H]RNA. AMV [³H]RNA (8000 cpm; 30 ng) was hybridized to a 6000-fold excess of total nuclear DNA (■), ds-DNA (▲), or ss-DNA (●) from AMV productive myeloblasts. AMV [³H]RNA (20,000 cpm, 75 ng) was hybridized to a 40,000-fold excess of total nuclear DNA from myeloblasts (□) or normal embryonic chicken cells (○). Aliquots were treated with pancreatic RNase (50 μg/ml) and T1 RNase (20 units/ml) and processed for trichloroacetic acid precipitation. Percentage hybridized was determined after subtraction of control values (time 0 or acid-precipitable fraction after thermal melting of the hybrid measured on other samples) that usually did not exceed 4–5% of the input [³H]RNA.

tion in the course of gene activity: one-third of the ss-DNA from normal embryonic chick cells (10) or human RD cells (11) could be hybridized to homologous RNAs.

As shown by hybridization kinetics, ss-DNA contains several thousand different transcription sites. It could therefore be postulated that ss-DNA must be enriched in DNA sequences corresponding to highly transcribed genes. Provirus DNA integrated in the genome of oncornavirus-transformed cells offers a good example of such genes. In fact, as shown above, AMV RNA and half of the AMV ds-cDNA sequences hybridized much faster to ss-DNA than to total DNA of AMV-productive myeloblasts and very little hybridized to ss-DNA of nonproductive cells. In contrast, very little of the AMV ss-cDNA hybridized to ss-DNA of myeloblasts. The reproducibility of these results permitted the conclusion that the ss-DNA from AMV-transformed cells is considerably enriched in virus-specific DNA fragments, mostly, if not exclusively, originating from the transcribed DNA strand. This raised the question of whether this selection of ss-DNA sequences is limited to tumor virus genes. It was reported that viral DNA binds preferentially to single strands of denatured host DNA (40), and, once integrated, single strand breaks can free the viral genome (41).

These data suggested a model for the replication of DNA tumor viruses, based on cellular and viral DNA single-strand interaction (42). In the case of Rous virus, nonintegrated provirus DNA molecules have been isolated from the cytoplasm of transformed cells, at a long time after infection (43). Although the occurrence of free DNA copies of the AMV genome in productively infected cells cannot be excluded, it is not likely that our results are due to such material. In preliminary experiments using nuclear DNA prepared by the Hirt procedure (44), similar results were obtained. The DNA pellet solubilized by moderate pipetting was analyzed by hydroxyapatite chromatography. Its ss-DNA content, which originally was about 0.6%, rose to 1.6% after further deproteinization and RNase treatment. Finally, at least two-thirds of the ss-DNA appeared to represent noncomplementary DNA fragments (see Fig. 1) which hybridize to bulk DNA and homologous RNAs (results not shown). The latter data suggest that not only the virus DNA sequences found in ss-DNA but also most of the ss-DNA fragments derive from the transcriptable DNA strand that codes for cellular mRNAs. This implies preferential single-strand breaks, occurring in the vicinity of DNA transcription sites either *in vivo* or in the course of DNA preparation.

This work was carried out with the skillful technical assistance of Mr. T. Huynh and support from Fondation pour la Recherche Medicale. S.A.L. is boursier of Ligue Nationale Francaise Contre le Cancer.

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# SOME EVIDENCE FOR REPLICATION-TRANSCRIPTION COUPLING IN *PHYSARUM POLYCEPHALUM*

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## SUMMARY

Hydroxyurea, at concentrations of 40–60 mM, selectively and effectively blocked incorporation of thymidine into DNA. Inhibition occurred within 5–10 min of application of the agent when DNA synthesis was in progress, while the onset of replication at the beginning of *S*-phase and DNA synthesis in $G_2$ phase were not affected.

Uridine incorporation into TCA-precipitable material, in the presence of hydroxyurea, was significantly (up to 70%) inhibited in early *S*-phase of the cell cycle. Selective inhibition of RNA synthesis was confirmed for RNA separated into rRNA-rich and poly-(A)-rich RNA fractions and analysed by the 2 kinds of DNA–RNA hybridization reactions. Uridine incorporation into poly (A) RNA was also inhibited under conditions where cycloheximide prevented maturation of nascent DNA molecules in early *S*-phase.

We assume that chromatin which is replicating early DNA sequences may be a more competent template for transcription.

## INTRODUCTION

In eucaryotic cells, doubling of the nuclear DNA content is a prerequisite for mitosis (Mitchison, 1971). Moreover, it could be argued that the sequence of DNA replication might contain a programme responsible for certain transition points of the cell cycle. Such a programme could be spelled out by the coupling of transcription of RNA with the temporal sequence of DNA replication. The synchronous cell cycle of *Physarum* (Rusch, 1970) lends itself to an investigation into the possibility of a coordinated replication and transcription, since there is good evidence for sequential replication during *S*-phase and some indication of differences in RNA during the cell cycle.

A temporal sequence of replication of *Physarum* DNA exists as demonstrated by density shift experiments (Braun, Mittermayer & Rusch, 1965). Ten steps, or rounds of replication, each dependent on the synthesis of a short-lived protein, have been concluded from experiments with cycloheximide (Muldoon, Evans,

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**EXHIBIT 2D**

Nygaard & Evans, 1971). Inhibition of DNA synthesis by cycloheximide might be due to blocking maturation of nascent DNA chains into large chromosomal DNA molecules (Brewer, 1972). Nuclear DNA from *Physarum* contains a significant amount of redundant base sequences and none of these seems to be replicated early in *S*-phase (Fouquet, Bierweiler & Sauer, 1974*b*). Therefore, qualitatively different DNA segments might be replicated at various points of the *S*-phase, starting perhaps with euchromatic portions of the chromatin.

Differences in the RNA composition during the cell cycle have previously been claimed after studying various parameters: the rate of uridine incorporation (Mittermayer, Braun & Rusch, 1964), base composition (Cummins, Weisfeld & Rusch, 1966) and nearest neighbour frequency distribution (Cummins, Rusch & Evans, 1967), DNA–RNA hybridization at high Cot values (Fouquet & Braun, 1974), the labelling pattern of poly (A) RNA (Fouquet *et al.* 1974*a*) and the residual RNA polymerase activities of isolated nuclei (Grant, 1972). Experiments with fluorodeoxyuridine (FUdR), an inhibitor of DNA synthesis affecting TMP synthesis, have led to the conclusion that in *Physarum*, the completion of the *S*-phase is an important step among the events that lead to mitosis (Sachsenmaier & Rusch, 1964). At the same time, FUdR has a direct effect on RNA synthesis, possibly after a conversion into FU, which can be counteracted by carefully adjusting the level of uridine in the growth medium (Sachsenmaier & Rusch, 1964).

In a first attempt to demonstrate a dependence of RNA synthesis on DNA synthesis, FUdR has again been used to block DNA synthesis. The results clearly indicate that in early *S*-phase, incorporation of deoxycytidine into DNA and RNA was significantly lower than in controls, even when enough uridine was added to overcome the direct effect of FUdR on RNA synthesis (Rao & Gontcharoff, 1969). We have begun to analyse further this possible correlation of DNA and RNA synthesis. We have used hydroxyurea and cycloheximide to inhibit DNA synthesis and have tried to determine which kinds of RNA become inhibited.

## MATERIALS AND METHODS

### *Preparation and labelling of cultures*

Mitotically synchronous macroplasmodia of *Physarum polycephalum* were set up as described elsewhere (Mittermayer, Braun & Rusch, 1965). Intermitotic time was 10 h. Experiments were performed between the second and third postfusion mitosis. The *S*-phase lasted 2·5–3 h and began in telophase. All times were expressed relative to metaphase, which lasted 5 min.

Labelling of macromolecules was done by adding tritiated precursors (uridine, adenosine, thymidine or leucine) with or without adding the agents to be tested (hydroxyurea, cycloheximide) to the growth medium. Incorporation of radioactive material was determined as we have previously described (Sauer, Babcock & Rusch, 1970).

### *Preparation and analysis of nucleic acids*

Nucleic acids were prepared from frozen plasmodia with a phenol method suitable for *Physarum* (Fouquet *et al.* 1974*a*). Gel electrophoresis was performed on these preparations without further steps of purification to minimize degradation of RNA molecules and to visualize the conversion of some uridine into DNA label.

Electrophoresis of 40 $\mu$g nucleic acids was carried out on 2·2% polyacrylamide gels at

8 mA/gel for 2 h at 14 °C (Bishop, Claybrook & Spiegelman, 1967). O.D.$_{260}$ was continuously measured over the whole gel and radioactivity was determined in hydrolysed slices of 2 mm thickness by scintillation counting. Radioactivity patterns were normalized, excluding material that remained on the gel start.

Further purification of *Physarum* RNA from DNA and slime was achieved by DNase treatment and chromatography on hydroxyapatite, as reported earlier (Fouquet *et al.* 1974*a*).

Chromatographic separation on Sigma Cell 38 and oligo-(dT) cellulose of RNA into fractions of peak I (rich in ribosomal RNA) and peak II (rich in poly (A) RNA) was done as previously described in detail (Fouquet *et al.* 1974*a*).

We applied 2 DNA–RNA hybridization procedures. In the first experiments the amount of DNA was limiting in the hybridization mixture. DNA–RNA hybridization was performed in a liquid system (Nygaard & Hall, 1964). Conditions for the hybridization reaction were such that most of the single copy DNA would have reacted during the 2 weeks of incubation as described previously for *Physarum* (Fouquet & Braun, 1974). In one experiment 0·1 $\mu$g of DNA and equal amounts of radioactivity (about 25–50 $\mu$g) of RNA of peak I were added to the reaction mixture. (This RNA contained mainly ribosomal RNA and an unknown proportion of non-ribosomal RNA of 3 possible kinds: never to be polyadenylated, not yet polyadenylated or broken 5′ ends of poly (A) RNA).

In the other experiment approximately equal amounts of radioactivity of peak II RNA were present in each hybridization mixture together with 0·1 $\mu$g of denatured DNA. This RNA was prepared from 50–100 $\mu$g of total RNA and had no measurable optical density.

In the second kind of RNA–DNA hybridization reaction radioactive RNA was limiting (Melli *et al.* 1971). We used poly-(A)-rich RNA prepared by oligo (dT) cellulose chromatography from controls and plasmodia treated with hydroxyurea in *S*-phase or $G_2$ phase. Denatured DNA was present in an approximately $10^3$–$10^4$-fold excess and DNA was allowed to renature over a wide range of Cot values to gain an estimate of which DNA segments might have coded for the poly (A) RNA present in the reaction mixture. RNA hybrids were measured after RNase digestion. Percent hybridization was computed from control values (100 %) obtained for each point of the graph (see Fig. 4).

## CHEMICALS

[$^3$H]uridine (26 Ci/mmol), [$^3$H]adenosine (0·1 Ci/mol), [$^3$H]thymidine (28 Ci/mmol) and [$^3$H]leucine (47 Ci/mmol) were purchased from Amersham Buchler. Hydroxyurea and cycloheximide were obtained from Serva and Sigma Cell 38 from Sigma. All other chemicals were of analytical grade quality.

## RESULTS

### *Effects of hydroxyurea on DNA synthesis*

When hydroxyurea (40 mM) was added to the growth medium, together with [$^3$H]thymidine, we noted a significant reduction of thymidine incorporation in *S*-phase of the cell cycle (Fig. 1A). Inhibition became effective within 10 min. When plasmodia were preincubated with hydroxyurea for 15 min before the addition of [$^3$H]thymidine, a more complete inhibition of thymidine incorporation was immediately observed (Fig. 1A). A mixture of deoxynucleosides (each at 1 mM), added together with hydroxyurea and [$^3$H]thymidine, resulted in no measurable inhibition of DNA synthesis (Fig. 1A).

We then studied the effect of increasing concentrations of hydroxyurea on DNA synthesis (Fig. 1B). At low concentrations (3·5 mM) of the compound we noted a significant stimulation of thymidine incorporation over that seen in controls.

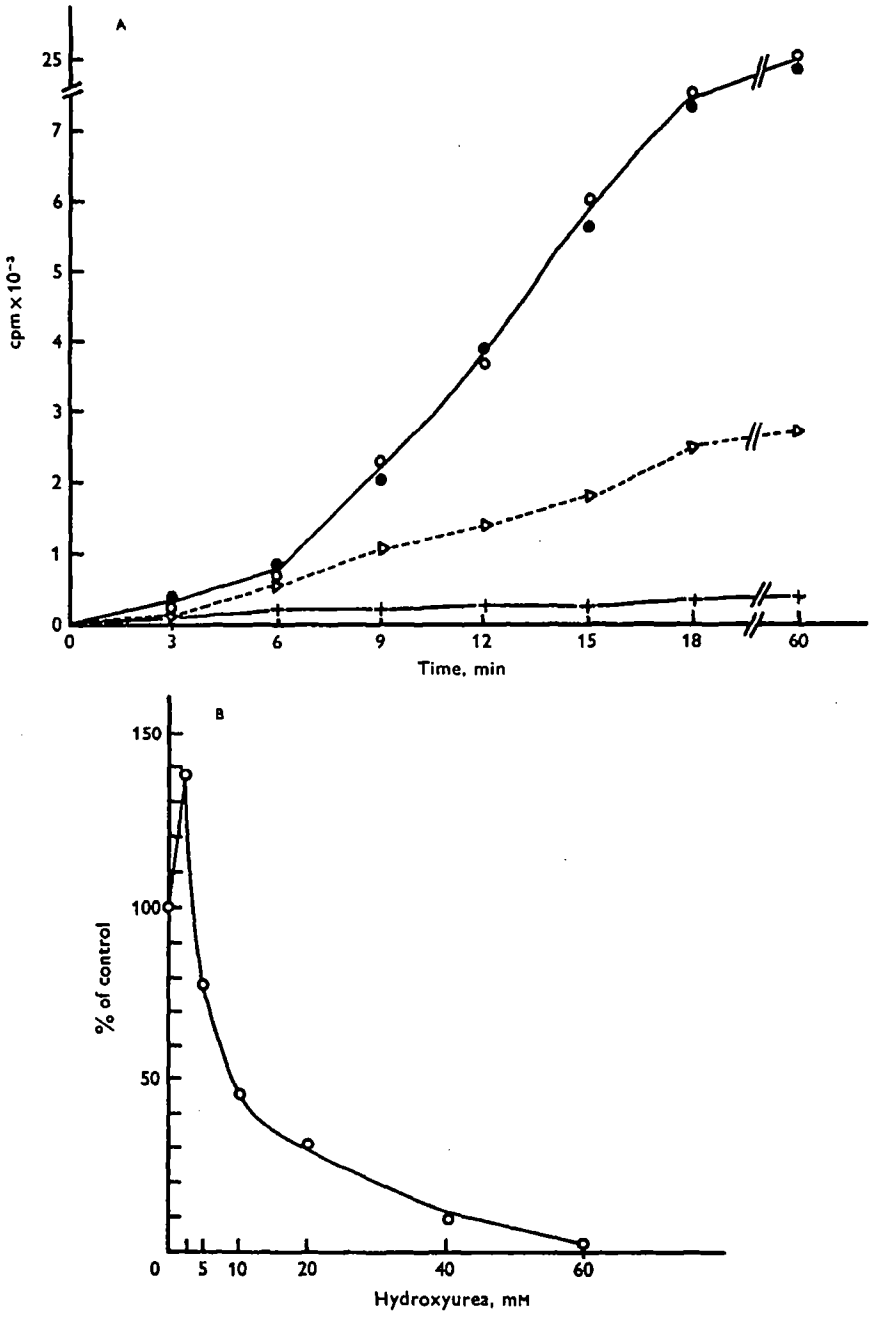


Fig. 1A and B. For legend see opposite.

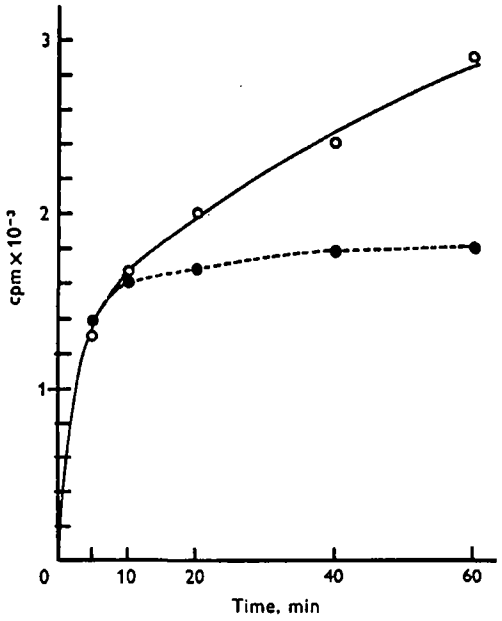


Fig. 2. Effects of hydroxyurea at the onset of DNA synthesis in *S*-phase of the cell cycle. Incorporation of [$^3$H]thymidine (10 $\mu$Ci/ml; cpm/mg protein); start was at telophase (10 min after metaphase). ○, control; ●, hydroxyurea (60 mM) added to the growth medium at 3·5 h before mitosis.

Inhibitions of 50 and 90 % were measured at 10 and 40 mM hydroxyurea, respectively. In the following experiments we applied this agent in appropriate concentrations (40–60 mM) to ensure blocking of DNA synthesis. At these concentrations protein synthesis, as measured by leucine incorporation (not shown), was not affected over a test period of 2 h.

In the next experiment we studied the effect of hydroxyurea on the initiation of chromosomal DNA replication, which took place in telophase of mitosis. We noted that, even after a long preincubation (up to 3·5 h in 30 or 60 mM hydroxyurea),

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Fig. 1. Effect of hydroxyurea on DNA synthesis.

A. Plasmodia were incubated for 60 min with [$^3$H]thymidine (10 $\mu$Ci/ml), beginning 30 min after the second postfusion mitosis. Aliquots for each experimental condition were from one macroplasmodium. Incorporation into TCA-precipitable material was determined (cpm/mg protein). ○, control; △, hydroxyurea (40 mM, in growth medium) was added at the same time as [$^3$H]thymidine; +, hydroxyurea (40 mM in growth medium) was added 30 min before [$^3$H]thymidine; ●, hydroxyurea (40 mM in growth medium), a mixture of deoxynucleotides (1 mM each, dA, dC, dG) and [$^3$H]thymidine were added simultaneously.

B. [$^3$H]thymidine incorporation was determined as in (A) over a period of 45 min in a control plasmodium (100 %) and in other plasmodia treated with various concentrations of hydroxyurea (percentage of control).

thymidine incorporation began as in controls (Fig. 2). However, after 10 min the incorporation had ceased almost completely.

Some DNA fractions, mitochondrial and nucleolar (approx. 10% of total DNA), are synthesized also in $G_2$ phase. Thymidine incorporation in $G_2$-phase into DNA was not affected by hydroxyurea (60 mM) present over a period of 2 h (from 6 to 8 h post mitosis, *M* II).

*Effects of hydroxyurea on RNA synthesis*

Hydroxyurea in sufficiently high concentrations to block DNA synthesis (40 mM) had little inhibitory effect (approx. 10%) on [$^3$H]uridine incorporation into material precipitable in 5% trichloroacetic acid (TCA), in $G_2$-phase (Fig. 3A). However,

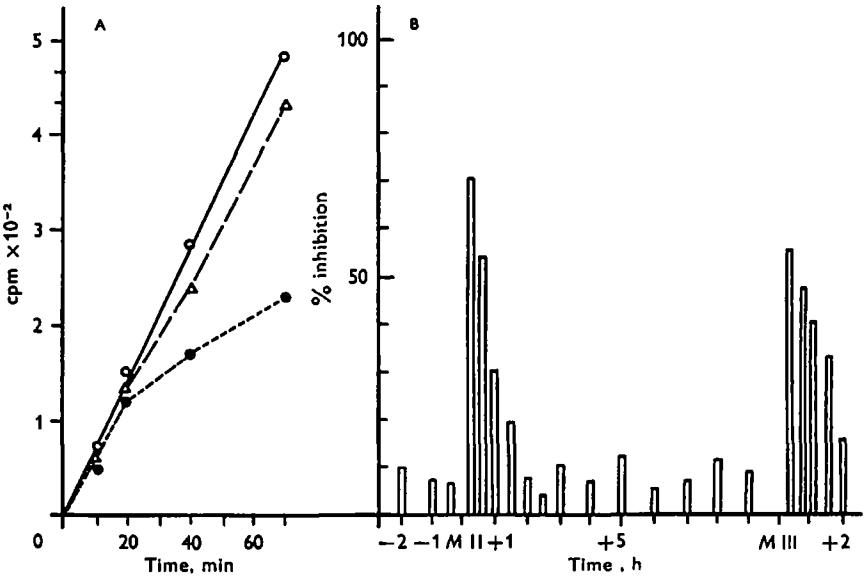


Fig. 3. Effects of hydroxyurea on uridine incorporation.

A. Plasmodia were incubated in [$^3$H]uridine (2 μCi/ml medium), and incorporation into TCA-precipitable material (cpm/μg protein) was measured. ○, control; △, hydroxyurea (40 mM) in $G_2$ phase (start was at 5 h post mitosis); ●, hydroxyurea (40 mM) in *S*-phase (start was 25 min post metaphase).

B. At several points of the cell cycle, ranging from 2 h before *M* II until 3 h after *M* III, the influence of hydroxyurea on uridine incorporation was determined as in Fig. 3A. The percentage of inhibition was computed from the incorporation values after 60 min.

significant inhibition of uridine incorporation was observed when the same experiment was done with a plasmodium treated with hydroxyurea in early *S*-phase (Fig. 3A). These experiments were repeated at many points of the cell cycle ranging from 2 h before mitosis (*M* II) until 3 h after the next mitosis (*M* III). Then the percentage of inhibition

of incorporation was computed from curves like the ones seen in Fig. 3A. Controls and experimental samples for each curve were taken from one macroplasmodium.

On comparing the effect of hydroxyurea at various stages of the cell cycle (Fig. 3B) we noted some inhibition during $G_2$ phase (approx. 10%), a high degree of inhibition in early *S*-phase (maximally 70%) and a significant inhibition in the middle part of *S*-phase (about 20–30%).

Next we studied the effects of hydroxyurea on nucleic acids extracted from plasmodial homogenates. Plasmodia were incubated for 30–120 min with [$^3$H]uridine in the presence of hydroxyurea (40 mM). Inhibition of incorporation ranged from 5–20% for $G_2$ phase and late *S*-phase plasmodia and amounted to 55% in early *S*-phase plasmodia. Therefore, the degree of inhibition measured in homogenates (TCA-precipitable material) and in extracted nucleic acids was quite similar.

Total nucleic acids, labelled for 30 min with [$^3$H]uridine, were analysed on 2·2% polyacrylamide gels. In all samples we detected low-molecular-weight material (4 S RNA), 19 S and 26 S ribosomal RNA and some material near the gel start (at about 1 cm) in the optical density profile. No measurable differences in these profiles from plasmodia prepared in *S*-phase or $G_2$ phase, treated or untreated with hydroxyurea, were observed.

There were some differences in the radioactivity patterns, aside from the general decrease in labelling of samples taken from plasmodia treated with hydroxyurea, and a fraction (approx. 20%) of radioactive material which had not entered into the gel.

We detected a distinct radioactive peak corresponding to the O.D. peak close to the gel start in untreated *S*-phase plasmodia. This radioactive material was shown to be DNA, since it was stable in 1 M NaOH, hydrolysed by 0·5 M perchloric acid (PCA) at 70 °C, insensitive to RNase but sensitive (over 90%) to DNase. Therefore, in *S*-phase some radioactivity after labelling with [$^3$H]uridine (approx. 10–15%) was found in DNA.

In addition, minor differences were observed in normalized radioactivity patterns obtained for early *S*-phase and $G_2$ phase. Half of each plasmodium served as control while the other half had been treated with hydroxyurea. After 30 min of labelling, no mature ribosomal RNA peaks were seen in the radioactivity patterns and 14·5 or 9·1% of the radioactivity was localized in the 4–30 S region (the presumptive mRNA region) of the gels from *S*-phase or $G_2$-phase samples, respectively. From plasmodia treated with hydroxyurea we obtained a value of 8·8% for blocked early *S*-phase and 9% for $G_2$-phase samples.

In the following experiments nucleic acids were treated with DNase. RNA was further purified by adsorption to and elution from hydroxyapatite. RNA was then separated into a fraction not absorbed at high ionic strength on a cellulose column (peak I RNA) and another fraction which eluted at low ionic strength (peak II RNA).

In Expt. 1 (Table 1) we compared the effect of hydroxyurea on uridine incorporation over 60 min into RNA of peaks I and II in the beginning of *S*-phase (5–65 min after mitosis, *M* II) with that seen in $G_2$ phase (5 h after mitosis; *S*-phase lasted 2·5–3 h). In $G_2$ phase we detected no inhibition of RNA synthesis in peak I or II.

However, significant inhibition was noted in early *S*-phase, 45 % of peak I RNA and 51 % of peak II RNA. In the next experiment (Table 1, Expt. 2) plasmodia were preincubated for 30 min with hydroxyurea and then labelled, together with controls, for 40 min with [$^3$H]uridine in early *S*-phase (15–55 min post mitosis, *M* II). The same incubation procedure was repeated in $G_2$ phase (beginning 5 h after mitosis, *M* II). RNA was separated in peaks I and II and aliquots of these RNA fractions were precipitated with TCA. We obtained 22 % inhibition of peak I RNA in *S*-phase (Table 1) and no inhibition in $G_2$ phase. Peak II RNA was inhibited by 74 % in *S*-phase and 21 % in $G_2$ phase.

Table 1. *Effects of hydroxyurea on purified RNA fractionated by cellulose chromatography*

| | | RNA synthesized (cpm/100 μg of total RNA) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Peak I RNA | | | Peak II RNA | | |
| Expt. no. | Cell cycle phase | Control | Hydroxy-urea | % inhibition | Control | Hydroxy-urea | % inhibition |
| 1 | *S* | 767622 | 421292 | 45 | 26677 | 12988 | 51 |
| 2 | *S* | 154360 | 120675 | 22 | 19823 | 5137 | 74 |
| 3 | *S* | 207741 | 195676 | 6 | 20113 | 8065 | 60 |
| 1 | $G_2$ | 732743 | 741607 | 0 | 8868 | 9410 | 0 |
| 2 | $G_2$ | 107201 | 105665 | 1 | 20244 | 16061 | 21 |
| 3 | $G_2$ | 244539 | 278581 | 0 | 10859 | 8445 | 22 |
| | | RNA hybridized (cpm/0·1 μg DNA) | | | | | |
| 2 | *S* | 1600 | 1129 | 30 | 647 | 240 | 63 |
| 2 | $G_2$ | 1110 | 909 | 8 | 411 | 334 | 18 |

For hybridization experiments, equal amounts of radioactivity (approx. 110000 cpm for peak I RNA and 15000 cpm for peak II RNA from each sample in *S*-phase and in $G_2$ phase treated or untreated with hydroxyurea) were incubated with denatured DNA. For both classes of RNA, hybridization values were higher (30 %) in the control samples from *S*-phase (Table 1). RNA from plasmodia treated with hydroxyurea in $G_2$ phase hybridized somewhat less than controls (8 against 18 %). Significantly less hybridization had occurred with RNA prepared from *S*-phase plasmodia in which DNA synthesis was blocked by hydroxyurea, by 30 % for peak I RNA and 63 % for peak II RNA (Table 1). In other experiments (not shown) plasmodia were labelled with [$^3$H]uridine in the second or third hour of the *S*-phase in the presence or absence of hydroxyurea. Inhibition of both classes of RNA ranged from 17 to 28 % in plasmodia treated with hydroxyurea.

In some cases plasmodia were labelled with [$^3$H]adenosine. From one representative experiment (Table 1, Expt. 3), after 60 min of labelling with [$^3$H]adenosine, we observed a strong inhibition of peak II RNA, while peak I RNA was almost not affected. This result was obtained in early *S*-phase. In $G_2$ phase measurable inhibition

of peak II RNA was observed but no inhibition of peak I RNA. The higher values of peak I RNA from $G_2$-phase plasmodia treated with hydroxyurea were not considered as evidence for a stimulatory effect of hydroxyurea.

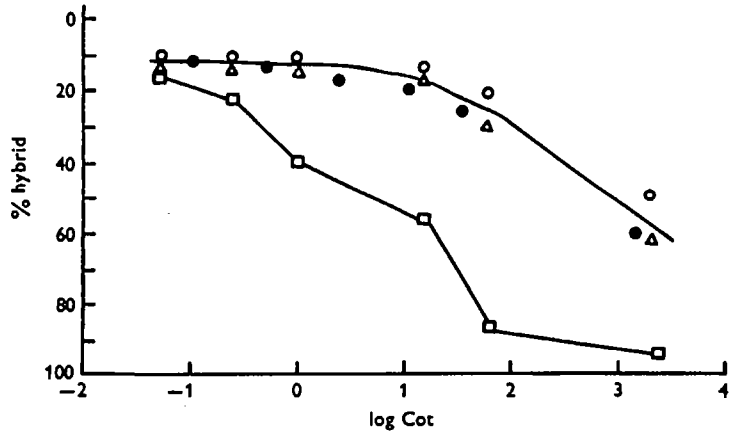


Fig. 4. Hybridization of poly (A) rich RNA with DNA in excess. Poly (A) RNA was prepared by affinity chromatography on oligo (dT) cellulose from control plasmodia in *S*-phase (●), in $G_2$ phase (○) and from plasmodia treated with hydroxyurea for 1 h in early *S*-phase (□) (10 min after *M* II) and $G_2$ phase (5 h post *M* II) (△). Plasmodia had been labelled for 50 min with 100 $\mu$Ci/ml of [$^3$H]-uridine and $2{\cdot}5-5 \times 10^4$ cpm of RNA were incubated with 1 mg/ml of DNA. Cot values were computed from the DNA concentration and incubation time.

For the following hybridization experiments poly (A) rich RNA (peak II RNA) was prepared by affinity chromatography from 4 macroplasmodia. Two plasmodia served as *S*-phase and $G_2$-phase controls. The other 2 plasmodia were treated with hydroxyurea (60 mM). In the DNA-driven hybridization reaction we did not detect major differences in the kinetics of DNA-RNA hybridization when the poly (A) RNA was prepared in *S*-phase or $G_2$ phase or from a $G_2$-phase plasmodium treated with hydroxyurea (Fig. 4). Only a small portion (10–20 %) of poly (A) RNA had reacted at a Cot value of 15. A substantial part of this RNA (50–60 %) had hybridized at a Cot value of 2000. Reannealing of single-copy DNA segments occurred under these conditions as was previously shown (Fouquet *et al.* 1974*b*).

Poly (A) RNA from a plasmodium blocked in early *S*-phase by hydroxyurea behaved differently. When compared with the samples prepared from *S*-phase controls or $G_2$-phase plasmodia (Fig. 4), this RNA had hybridized to a significant degree (40 %) already at a low Cot value of 1 and had reacted almost to completion at a Cot value of 70.

*Effects of cycloheximide on RNA synthesis*

Plasmodia were incubated with cycloheximide (5 $\mu$g/ml growth medium) for 20 or 60 min in the presence of [$^3$H]uridine (Table 2). Purified RNA was fractionated

into peaks I and II by cellulose chromatography. We detected no significant differences in the RNA fractions analysed in $G_2$ phase (maximum inhibition was 11 %). However, inhibition of [$^3$H]uridine incorporation into both RNA fractions, especially into peak II RNA, was seen when the experiment was done in early *S*-phase. The extent of inhibition of labelling of both RNA species was similar to that seen with hydroxyurea.

Table 2. *Effect of cycloheximide on RNA synthesis in Physarum*

| Cell cycle phase | Incubation with [$^3$H]uridine, min | % inhibition | |
|---|---|---|---|
| | | Peak I RNA | Peak II RNA |
| *S* | 20 | 30 | 70 |
| *S* | 60 | 32 | 55 |
| $G_2$ | 20 | 0 | 11 |
| $G_2$ | 60 | 0 | 0 |

## DISCUSSION

Hydroxyurea (above 40 mM) was shown to block almost completely DNA synthesis in *Physarum*. Our results confirmed an earlier report of a stimulation of thymidine incorporation by low concentrations of hydroxyurea, and inhibition at a higher concentration and its reversal by deoxynucleosides (Evans, Menz & Nygaard, 1968). Therefore, in *Physarum*, hydroxyurea might inactivate ribonucleotide reductases as in other systems and consequently limit the supply of DNA precursors (Adams & Lindsay, 1967; Steinert, 1969). From an analysis of pool sizes in plasmodia from *Physarum*, a DNA synthesis time ranging from 1·5 min (for dATP) to 13·8 min (for dTTP) had been computed (Bersier & Braun, 1974*a*, *b*). This calculation fitted the time span observed for a significant inhibition of thymidine incorporation (5–10 min) and was consistent with the fact that initiation of replication (at telophase of mitosis) was not prevented even after long preincubation with hydroxyurea.

In early *S*-phase of the cell cycle of *Physarum*, uridine incorporation into TCA-precipitable material was significantly reduced in the presence of hydroxyurea. This finding confirmed earlier results obtained with FUdR, another indirect inhibitor of DNA synthesis (Rao & Gontcharoff, 1969). A portion of the inhibition measured in this way (approx. 15 %) was an artifact due to labelling of DNA with uridine, possibly after conversion into cytosine (Evans, cited by Schiebel, 1973). A small fraction of low-molecular-weight RNA might also be directly involved in replication and thus also contribute to the differences in labelled RNA seen after a blockade of replication (Waquar & Huberman, 1973).

On comparing the effect of hydroxyurea on early *S*-phase plasmodia with that seen in later periods of *S*-phase and $G_2$ phase, we noted a significant reduction of 2 labelled RNA classes (peak I RNA, rich in ribosomal RNA, and peak II RNA, rich in poly (A) RNA), and a possible decrease of radioactive RNA fractions in the

mRNA region of gels. A similar degree of inhibition of the 2 RNA classes was found after a blocking of DNA replication with cycloheximide.

According to DNA-RNA hybridization experiments, both RNA classes extracted from plasmodia which had been labelled in early *S*-phase in the presence of hydroxyurea, hybridized to a smaller extent than controls. These results might indicate qualitative changes in the RNA composition, although the specific activities of the heterogeneous RNA preparations, which would allow an estimate of missing RNA species, were not known. The DNA-driven hybridization reaction with poly (A) RNA clearly indicated distinct differences when this RNA component was prepared from plasmodia treated with hydroxyurea in early *S*-phase. This RNA, contrary to controls, lacked practically all the material reacting with single copy DNA and contained mainly material which reacted with DNA of moderately redundant base sequences (Fouquet *et al.* 1974*b*).

The changes in RNA composition, both quantitative and qualitative and especially for peak II RNA (containing mRNA), observed in correlation with blockage of DNA synthesis, might constitute evidence for replication–transcription coupling.

Since replicating DNA of *Physarum* contained many unjoined pieces in *S*-phase, RNA polymerase B might preferentially initiate transcription at such open DNA structures. As a consequence, sequential replication would determine sequential transcription in *S*-phase. This possibility would then support a linear reading model of transcription (Tauro, Halvorson & Epstein, 1968) and might help to explain the temporal sequence of such events which are of significance in the DNA division cycle (Mitchison, 1971).

However, up to now there was no evidence for the newly replicating DNA being the template for immediate transcription. Preliminary DNA–RNA hybridization experiments with density-labelled early DNA at low Cot values were negative (Zellweger & Braun, 1971). Ribosomal DNA which was postulated to be among the early DNA segments to be replicated, according to a recent experiment with FUdR (Gontcharoff & Rao, 1972), was clearly shown to replicate at any time of the cell cycle but the early *S*-phase (Guttes, 1974).

Therefore, coupling of RNA synthesis with replication might also be explained by a change in chromatin structure in early *S*-phase, rendering some DNA segments more competent for transcription.

Since poly (A) RNA is more severely inhibited, it could be argued that the presumed replication-dependent inhibition might also be explained by a direct effect of hydroxyurea on poly (A) RNA synthesis. Then the smaller effect of hydroxyurea in $G_2$-phase plasmodia might be explained by an assumption that, in $G_2$ phase, there was generally less RNA polymerase B activity in plasmodia. Therefore, hydroxyurea might specifically inhibit RNA polymerase B or interfere somehow with polyadenylation.

Isolated RNA polymerases A and B from *Physarum* (Hildebrandt & Sauer, 1973) when assayed *in vitro* were almost completely inactive in the presence of 5 mM hydroxyurea. Enzyme A, however, seemed to be more sensitive than enzyme B (unpublished experiments from our laboratory). On the other hand, the inhibitory

effect of hydroxyurea on poly (A) RNA synthesis *in vivo* was also small in late *S*-phase (only 17–28%) when, according to the experiments with isolated nuclei (Grant, 1972), RNA polymerase B was shown to be at peak activity.

A direct effect of hydroxyurea on polyadenylation, responsible for some inhibition of the formation of poly (A) RNA, however, could be concluded from the relatively higher inhibition of peak II RNA in both *S*-phase and $G_2$ phase, labelled with [$^3$H]adenosine instead of [$^3$H]uridine.

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# $S_1$ nuclease definition of highly repeated DNA sequences in the Guinea pig, Cavia porcellus

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ABSTRACT

Native DNA of the Guinea pig, Cavia porcellus, purified from liver or tissue culture cells, was heat denatured and reassociated to a $C_0t$ value of 0.01 (equivalent $C_0t$ value of $7.2 \times 10^{-2}$). The reassociated DNA was isolated by digestion with the single-strand DNA specific enzyme $S_1$ nuclease. Spectrophotometric and radioactivity assays demonstrated that 24% of the total DNA was resistant to $S_1$ nuclease treatment. Zero-time reassociation indicated that approximately 3% of the DNA was inverted repeat sequences. Thus, highly repeated sequences comprised 21% of the total genome. CsCl buoyant density ultracentrifugation indicated that this fraction was composed of both main band and satellite sequences. Although actinomycin D - CsCl density gradients failed to give significant separation of the repetitive sequences, distamycin A - CsCl gradients were able to fractionate the DNA into several overlapping bands. The heterogeneity of the repetitive DNA was further demonstrated by the first derivative plots calculated from their thermal denaturation profiles. This analysis revealed six major thermalytes which indicate that there may be at least six discrete components in the repetitive DNA.

## INTRODUCTION

Investigations of Guinea pig DNA have demonstrated that three highly repeated satellite sequences exist which, in CsCl density gradients, are slightly denser than the main band DNA (1). Satellite I has been shown to consist of the hexanucleotide sequence 5'-CCCTAA-3' (2). Although satellites II and III may have been derived from the same ancestral sequence as satellite I, they appear to be more complex (3). No molecular studies, however, have been carried out to determine whether highly repeated sequences are present in the main band region of CsCl or $Ag^+Cs_2SO_4$ density gradients, but are obscured by the bulk of the non-repeated DNA.

Unfortunately, in only two (4,5) of the many molecular studies conducted on Guinea pig DNA was the taxonomy of the species identified. As illustrated by studies of the genera Mus (6), Drosophila (7) and Dipodomys (8), closely related species may have different amounts of





EXHIBIT 2E

the same highly repeated satellite or completely different satellites. In the Guinea pigs, genus Cavia, nearly 20 species have been on record, and two of them, Cavia porcellus and C. cobaya, are used in clinical and biological laboratories. Most investigators working with Guinea pigs simply refer to the common name or assign a species name without taxonomic verification. Thus reported data may represent the mixture of at least two different species. We have examined the DNA of one particular species of Guinea pig, C. porcellus, defined by its karyology (9,10), and report some of the physical properties of the total complement of the repeated DNA sequences. The chromosomal locations of these same sequences, as demonstrated by in situ hybridization have previously been reported (10).

## MATERIALS AND METHODS

### DNA preparation

Guinea pig livers were removed and quickly frozen on dry ice. The DNA was isolated by the Marmur method (11), with several modifications. The frozen tissue was sliced and homogenized in saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The nuclei were lysed by the addition of pronase (200 µg/ml; CalBiochem) and SDS (final concentration 0.13%; Matheson Coleman and Bell). After overnight incubation at 37°C, the solution was deproteinized by shaking with redistilled phenol saturated with saline-EDTA. After centrifugation, the DNA was precipitated from the aqueous layer with 95% ethanol and redissolved in 0.1 X SSC. The salt concentration was then raised to 1 X SSC. Pancreatic RNase (50 µg/ml; type I-A, Sigma), $T_1$ RNase (300 U/ml, Sigma) and α-amylase (100 µg/ml; Bacillus subtilis type II-a, Sigma) were added and the mixture was incubated at 37°C for 90 min. Pronase (100 µg/ml) was added and the incubation was continued for 1 hr. The mixture was deproteinized with a solution of equal parts SEVAGS (chloroform-isoamyl alcohol, 24:1) and saturated phenol. After centrifugation, the DNA was precipitated and redissolved, as above. Sodium acetate-EDTA (2.0 M sodium acetate, 1 mM EDTA; 0.1 volume) was added to the DNA and the DNA was precipitated with 0.54 volume of 2-propanol. The DNA was redissolved in 0.1 X SSC. Each liver yielded about 20 mg of DNA.

An aliquot of DNA was dialyzed against 1.0 M NaCl, 10 mM Tris, pH 8.0 buffer and 0.1 volume of 1.0 N NaOH (12) was then added. The molecular weight of the denatured DNA was determined by velocity sedimentation in a Beckman Model E analytical ultracentrifuge equipped with an automatic ultraviolet scanner.

Tissue culture

Fibroblast cell lines were derived from ear biopsies of the same animals from which the liver tissue was obtained. The cultures were maintained in a modified McCoy's 5a medium supplemented with 20% fetal calf serum (GIBCO). Confluent cultures were subcultured by detachment of the cells from the culture flask with a 0.01% trypsin (3X purified; Worthington) solution and replating the cells into two or more flasks.

Preparation of tritium labeled DNA

Radioactive Guinea pig DNA was isolated from tissue culture cells. Tritiated thymidine (0.5 μCi/ml, specific activity 6.7 Ci/mM; New England Nuclear) and unlabeled thymidine (10.0 μg/ml; CalBiochem) were added to tissue cultured cells in exponential growth. After 54 hr, the cells were washed with saline-EDTA, detached from the flasks with 0.1% pronase and centrifuged. The cell pellet was resuspended in saline-EDTA, and pronase (50 μg/ml) and SDS (final concentration 0.25%) were added. The mixture was incubated and the phenol extraction was performed as described above. The specific activity of the [$^3$H]DNA was 1250 cpm/μg.

Assay of $S_1$ nuclease activities

The *Aspergillus oryzae* $S_1$ nuclease purified from takadiastase powder (Sankyo) by DE-52 and G-100 column chromatography (13) was assayed for single-strand activity by using 15 μg of a heat denatured mixture of [$^3$H]*B. subtilis* DNA (5000 cpm/μg) and non-radioactive human placental DNA (1:14) as substrate. The reaction was carried out in $10^{-2}$ M NaCl, $3 \times 10^{-2}$ M sodium acetate and $3 \times 10^{-5}$ M $ZnCl_2$, pH 4.5, in a total volume of 0.5 ml. After incubation of 50°C for 10 min, the reaction was stopped by the addition of 25 - 50 μg of nonradioactive carrier DNA and an equal volume of 10% trichloroacetic acid (TCA; Fisher). The TCA precipitable material was loaded onto glass fiber filters (Reeve Angel) with gentle suction and washed with 5% TCA and then 95% ethanol. The filters were counted in a Beckman LS-100C scintillation counter. One unit of activity was defined as the amount of enzyme solubilizing 1% of the denatured [$^3$H]DNA in 10 min (13). The specific activity of the enzyme preparation was 9.9U/μg. The protein concentration was measured by the Folin phenol reagent method (14).

The enzyme was assayed for double-strand activity using unlabeled bacteriophage PM2 DNA or tritium labeled polyoma DNA (specific activity 24,000 cpm/μg). The polyoma DNA was prepared by the Hirt procedure, as described in Fried (15), using a low multiplicity infection of secondary

mouse embryo cells in the presence of tritiated thymidine. The PM2 DNA was incubated in the $S_1$ buffer with 104 U $S_1$ at 50°C for 10 min. The reaction was stopped by chilling in an ice water bath and adding EDTA to a final concentration of 20 mM. The PM2 digestion mixture was layered on 1.4% agarose (Bio-Rad) gels and electrophoresed in E buffer (40 mM Tris-HCl, pH 7.9, 5 mM sodium acetate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide (16). Molecular weight markers for the 1.4% agarose gels were generated by digestion of the PM2 DNA with either Hpa II (16) at 37°C for 2 hr or Hind III (17) restriction enzymes. The Hind III digestion was carried out in 6 mM Tris, pH 7.5, 6 mM $MgCl_2$ and 40 mM NaCl at 37°C for 24 hr. The [$^3$H]polyoma DNA was incubated at similar enzyme to DNA ratios and the TCA precipitations were washed and counted as previously described.

Isolation of repeated DNA sequences

Native Guinea pig DNA, with an average length of 15,500 $\pm$ 600 base pairs, was heat denatured and immediately added to buffer kept at 60°C (final salt concentration 1 X SCC). The DNA was reassociated to a $C_o t$ value of 0.01, which corresponds to an equivalent $C_o t$ value of $7.2 \times 10^{-2}$ (18). The reassociation was stopped by chilling to 4°C and 15 - 20 µg aliquots of DNA were added to $S_1$ buffer with 104 U $S_1$ (final volume 0.5 ml). The digestion was carried out for 10 min at 50°C and then stopped by placing the mixture into an ice water bath. The unlabeled DNA was dialyzed extensively into 1 X SSC.

The amount of renatured DNA remaining after digestion was determined by either TCA precipitation and scintillation counting of the [$^3$H]DNA or by spectrophotometry of the unlabeled DNA. Because of the significant ultraviolet absorbance of the $S_1$ at 260 nm, a set of simultaneous equations were derived whose solution allows the determination of the DNA absorbance (A) at 260 nm in the presence of the protein.

If we assume that the absorbance of nucleic acids (DNA) and proteins (P) are additive at both 260 and 280 nm, then

$$A_{260} = A_{260}^{DNA} + A_{260}^{P} \text{ and} \quad [1]$$

$$A^{280} = A_{280}^{DNA} + A_{280}^{P} \quad [2]$$

where $A_{260}$ and $A_{280}$ are the final observed absorbance at 260 and 280 nm, respectively. If we also define the ratios

$$\alpha = A_{260}^{DNA} / A_{280}^{DNA} \text{ and} \quad [3]$$

$$\beta = A_{260}^{P} / A_{280}^{P} \quad [4]$$

then $A_{260}^{DNA} = A_{260} - A_{260}^{P} = A_{260} - \beta A_{280}^{P} = A_{260} - \beta(A_{280} - A_{280}^{DNA})$. [5]

Substituting α into the equation gives

$A_{260}^{DNA} = A_{260} - \beta A_{280} + \beta A_{260}^{DNA}/\alpha$. [6]

Solving for $A_{260}^{DNA}$ vives

$A_{260}^{DNA} = [A_{260} - \beta A_{280}]/(1 - \beta/\alpha)$. [7]

By independent determination of the ratios α and β we were able to determine the absorbance of the DNA at 260 nm in the presence of the enzyme. From the absorbance we were able to calculate the concentration of the double-stranded DNA in the unlabeled sample.

Analytical ultracentrifugation

DNA samples were examined by analytical ultracentrifugation in CsCl density gradients (42,040 rpm, 25°C, 20 - 24 hr). Bacteriophage φ2C DNA, $\rho = 1.742$ g/cm$^3$ (19), was used as a reference density marker. In gradients without a marker, the density of the DNA was calculated by the root-mean-square method (20), using β values interpolated from the data of Ifft et al. (21). The ultraviolet absorbance at 265 nm was recorded on Kodak electron microscope film and the negatives were traced on a Joyce Loebl double beam recording microdensitometer, Model E12 MKIIIB.

Antibiotic - DNA density gradients

Actinomycin D (Cosmegen®, MW 1254.74; Merck, Sharp and Dohme) and distamycin A HCl (MW 517.96; Farmitalia) were suspended in buffer (1 X SSC or 0.025 M Tris HCl, pH 7.95), at final concentrations of 500 μg/ml and 1000 μg/ml, respectively. These antibiotics were added to both native and repetitive DNA samples at different $r_f$ values (range 0.33 - 2.0, w/w). The samples were then examined by analytical ultracentrifugation.

DNA denaturation analysis

Thermal denaturation was carried out in a Zeiss PM QII spectrophotometer equipped with an automatic recorder. Sample temperature was increased 0.5°C per min by a circulating water bath controlled by a Lauda linear temperature programmer. The melting temperature ($T_m$) was determined in either 0.1 X SSC or 1 X SSC.

The thermal denaturation curves were smoothed by connecting the midpoints between two consecutive data points. At each temperature point, the hyperchromicity was obtained from the constructed midpoint lines. After this smoothing, the change in hyperchromicity vs. change in temperature ($\Delta H/\Delta T$) was calculated and plotted at the temperature of the

corresponding midpoint.

## RESULTS

### Buoyant density analysis of Guinea pig DNA

In neutral CsCl buoyant density gradients, native C. porcellus DNA, isolated either from the liver (Fig. 1a) or cultured fibroblasts cells (Fig. 1b), displayed a density distribution similar to those previously reported (27-29). The density of the main band was 1.700 g/cm$^3$ and that of the satellite, which appeared as a shoulder on the dense side of the main band, was 1.703 g/cm$^3$. Although there might have been differences in the relative amounts of particular sequences, no new density classes were apparent. The profiles of the liver and fibroblast DNA were very similar.

After thermal denaturation at 100°C for 15 min, the liver DNA profile showed a characteristic density shift to 1.713 g/cm$^3$ (Fig. 1c). Upon

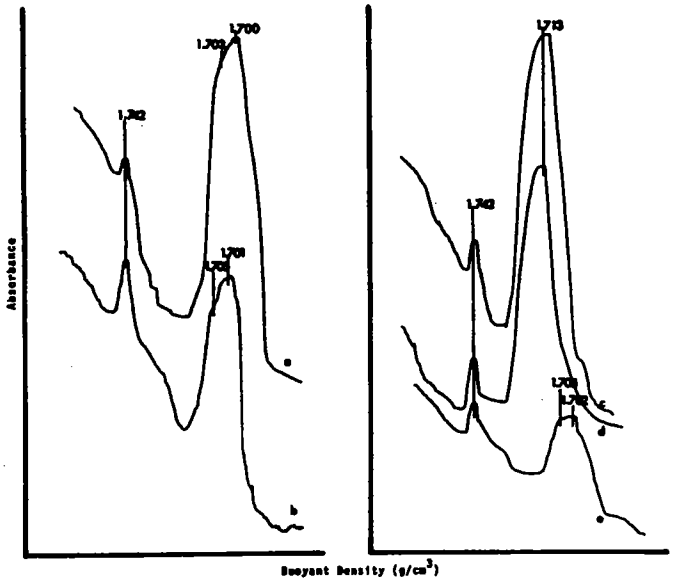


Figure 1. Analytical ultracentrifugation of Guinea pig DNA. DNA samples were centrifuged to equilibrium in CsCl at 42,040 rpm and 25°C for 20–24 hr. Bacteriophage $\phi$2C ($\rho$ = 1.742) was used as a density marker. a) native liver DNA, b) native fibroblast culture DNA, c) denatured liver DNA, d) denatured liver DNA reassociated to an equivalent $C_0t$ value of 7.2 x $10^{-2}$ and e) denatured liver DNA reassociated to an equivalent $C_0t$ value of 7.2 x $10^{-2}$ and then digested with 104 U $S_1$ nuclease.

reassociation to an equivalent $C_o t$ value of $7.2 \times 10^{-2}$, a small amount of the DNA was observed at the density of native DNA (Fig. 1d). When the reassociated sample was digested with $S_1$ nuclease, the remaining DNA had densities of 1.705, 1.702 and 1.701 $g/cm^3$ (Fig. 1e). No DNA was found in the region previously occupied by denatured sequences, indicating that the digestion went to completion. Tissue culture DNA, treated in the same manner as the liver DNA, behaved similarly in CsCl density gradients.

Quantitation of highly repeated sequences

Earlier studies have indicated that approximately 10% of the Guinea pig genome was contained in the three highly repeated satellites which were expected to be present in our reassociated fraction (1). This figure did not account for any highly repeated sequences present in the main band DNA. We have determined the total amount of our reassociated DNA by two different methods. The [$^3$H]DNA isolated from fibroblast culture cells was reassociated to an equivalent $C_o t$ value of $7.2 \times 10^{-2}$. After complete digestion of 17.06 µg of DNA with $S_1$ nuclease, 24.3% of the total DNA remained as TCA precipitable material (Table I).

Since the liver DNA could not be uniformly labeled for these experi-

TABLE I

RADIOACTIVITY ASSAY OF REASSOCIATED GUINEA PIG DNA

REMAINING AFTER $S_1$ NUCLEASE TREATMENT

| Post-Reassociation Treatment* | TCA Precipitable cpm | % TCA Precipitable cpm |
|---|---|---|
| A. Untreated | 21,100 | 100.0 |
| B. $S_1$ Nuclease | 5,300 | 25.1 |
| C. $S_1$ Nuclease | 5,110 | 24.2 |
| D. $S_1$ Nuclease | 5,000 | 23.7 |
| E. Average of values in B, C and D | 5,140 (S.D. $\pm$ 150) | 24.3 (S.D. $\pm$ 0.7) |

*All samples were denatured and reannealed to an equivalent $C_o t$ value of 0.072.

Results of experiments in B, C and D are independent $S_1$ nuclease digestions of different reassociated samples from the same DNA preparation.

ments, we developed a spectrophotometric method to measure the fraction of highly repeated sequences in this sample (see MATERIALS AND METHODS). In order to avoid DNA loss during the extraction of $S_1$ from the sample, the digestion mixture was extensively dialyzed against 1 X SSC and the optical density and volume of the dialysate was measured. Using the extinction coefficient of double-stranded DNA at 260 nm we found that, in three separate experiments, 24.1% of the DNA was present in the reassociated, highly repeated fraction (Table II).

The reassociated DNA fraction contained inverted repeat sequences. The quantity of these sequences was analyzed by assaying the amount of double-strand DNA present after denaturation and zero-time reassociation. The snap-back DNA constituted 2.7% of the total genome. Highly repeated sequences, therefore, comprised 21% of the total DNA.

Fractionation of the reassociated sequences by antibiotic-DNA density gradients

Our reassociated sample apparently was made up of several families of DNA sequences, as seen by its CsCl pattern (Fig. 1e) and large percentage

TABLE II

SPECTROPHOTOMETRIC ASSAY OF REASSOCIATED DNA REMAINING AFTER $S_1$ NUCLEASE TREATMENT

| Post-Reassociation Treatment* | Micrograms of Reassociated DNA+ | | % of DNA Resistant to $S_1$ Nuclease |
|---|---|---|---|
| | Before Digestion | After Digestion | |
| A. $S_1$ Nuclease | 1,220 | 274 | 22.5 |
| B. $S_1$ Nuclease | 419 | 110 | 26.3 |
| C. $S_1$ Nuclease | 193 | 45 | 23.6 |
| D. Average of experiments in A, B and C | - | - | 24.1 (S.D. $\pm$ 2.0) |

*All samples were denatured and reannealed to an equivalent $C_0t$ value of 0.072.

+Quantities of DNA were determined using equation [7], as described in MATERIALS AND METHODS, and a specific extinction coefficient of 50 µg per one absorbance unit at 260 nm.

Results of experiments in A, B and C are independently determined $S_1$ nuclease digestions of different reassociated samples from the same DNA preparation.

of the genome (Tables I and II). In order to examine the degree of heterogeneity, the DNA was complexed with antibiotics and analyzed in CsCl gradients. Actinomycin D, which intercalates preferentially into the GpC dinucleotides of double-stranded DNA (30), was complexed with native DNA in various antibiotic/DNA ratios ($r_f$, w/w). At ratios of 0.33, 0.50 or 1.00, the DNA was fractionated into a minor and a major component with buoyant densities of 1.689 and 1.671 g/cm$^3$, respectively (Fig. 2a). The reassociated sample did not display any fractionation at the same antibiotic/DNA ratios (Fig. 2b).

Distamycin A HCl, which preferentially binds to AT rich sequences (31), was used with greater success to separate different DNA classes in both the native and repetitive samples. At $r_f$ = 0.50, native Guinea pig DNA was fractionated into at least four components with buoyant densities of 1.622, 1.626, 1.637 and 1.648 g/cm$^3$ (Fig. 3a). The reassociated highly repeated sequences were also separated into several components with den-

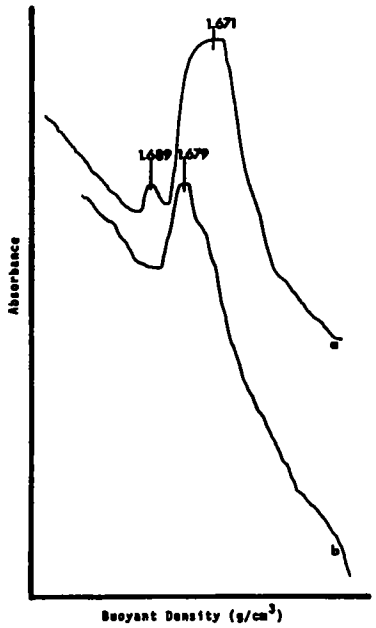


Figure 2. Actinomycin D-CsCl density gradients. Actinomycin D in either 1 X SSC or 0.025 M Tris HCl, pH 7.95, was added to DNA samples and the mixture was analyzed by CsCl buoyant density ultracentrifugation. a) native DNA, $r_f$ = 0.50, b) highly repeated sequences, $r_f$ = 0.50.

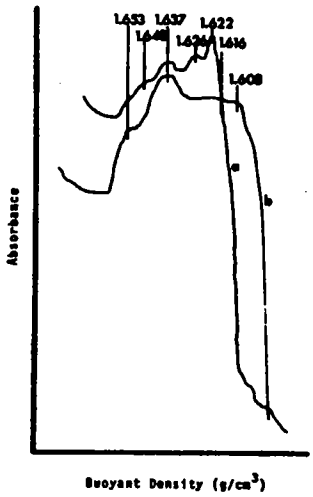


Figure 3. Distamycin A-CsCl density gradients. Distamycin A in either 1 X SSC or 0.025 M Tris HCl, pH 7.95, was added to DNA samples and the mixture was analyzed by CsCl buoyant density ultracentrifugation. a) native DNA, $r_f$ = 0.50, b) highly repeated sequences, $r_f$ = 0.50.

sities of 1.608, 1.616, 1.637 and 1.653 g/cm$^3$, at $r_f$ = 0.50 (Fig. 3b).

Thermal denaturation analysis

Heterogeneity can also be demonstrated by thermal denaturation profiles if the base composition of the various DNA families are sufficiently different. The denaturation profile of Guinea pig liver DNA possessed a single sharp transition when the denaturation was performed in either 0.1 X SSC ($T_m$ = 67.0°C; Fig. 4a) or 1 X SSC ($T_m$ = 82.6°C; Fig. 4b). In contrast, the denaturation profile of the reassociated highly repeated DNA displayed a broad transition in both 0.1 X SSC ($T_m$ = 65.3°C, Fig. 4a) and 1 X SSC ($T_m$ = 78.3°C; Fig. 4b). Both the native and repetitive DNA denaturation profiles showed a characteristic increase in their respective melting temperature and decrease in their transition breadths when the denaturations were performed in 1 X SSC as compared to 0.1 X SSC.

In both 0.1 X SSC and 1 X SSC, the reassociated repeated DNA displayed some stepwise melting, indicative of the presence of several different sequence or base composition classes of the DNA. Calculation of the first derivative of the denaturation hyperchromicity as a function of temperature in both 0.1 X SSC and 1 X SSC clearly revealed the heterogeneity of DNA

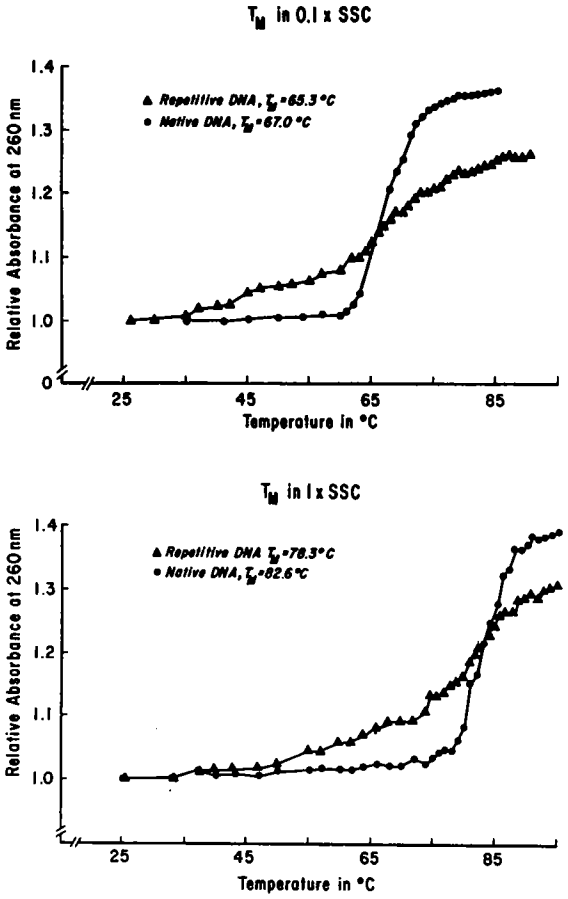


Figure 4. Thermal denaturation of Guinea pig DNA. The melting temperatures of native (●) and highly repeated (▲) DNA sequences were determined in a) 0.1 X SSC and b) 1 X SSC.

sequences in the reassociated repetitive fraction (Fig. 5a and b). In both 0.1 X SSC and 1 X SSC, we detected a minimum of six thermal transitions, reflecting the presence of at least six classes of repeated DNA sequences. It should be noted that the relative positions of these thermalytes may vary with ionic strength (32).

Characterization of $S_1$ nuclease activities

Before using the $S_1$ nuclease preparation to define the highly repeated sequences in the Guinea pig DNA, we examined two aspects of the enzyme digestion procedure which could significantly effect the interpretations

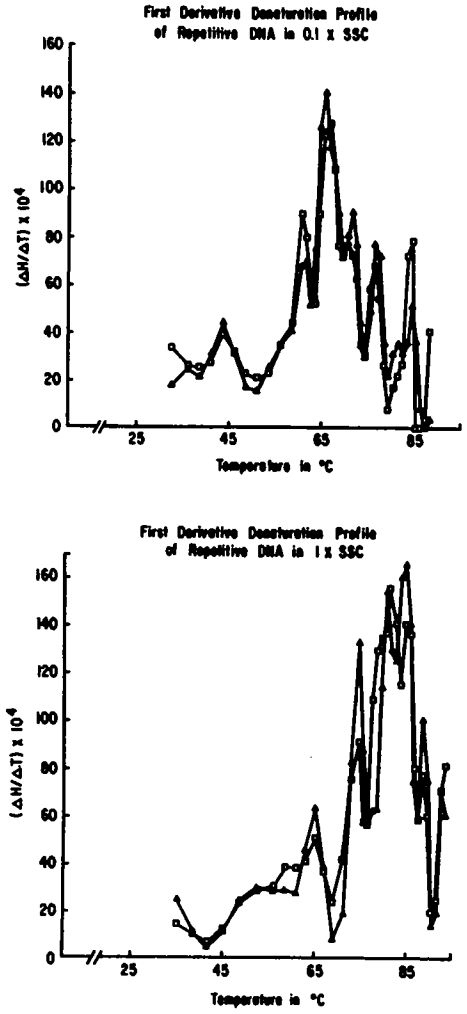


Figure 5. First derivative profiles of the thermal denaturation of Guinea pig highly repeated DNA. Thermal denaturation of the samples shown in Fig. 4 (▲) and of duplicate repetitive samples (□) were analyzed by the change in hyperchromicity as a function of the change in temperature in a) 0.1 X SSC or b) 1 X SSC.

of our results. Since crude preparations of $S_1$ nuclease possess double-strand deoxyribonuclease activity (22), we considered it necessary to test for such activity in our purified preparation. DNase activity was assayed using bacteriophage PM2 DNA. Covalently closed ciruclar (form

I) or nicked circular (form II) PM2 DNA should be converted to linear unit length duplex DNA (form III) by the single-strand specific nuclease activity of the enzyme, as seen in experiments done on SV40 (23) and polyoma (24) DNAs. Degradation of the linear duplexes to shorter lengths, however, would result from the double-strand DNase activity. Samples of PM2 DNA, which contained a mixture of form I and II DNAs, were incubated under the conditions used for the isolation of the reassociated repetitive Guinea pig DNA, and then electrophoresed in 1.4% agarose gels. In Fig. 6, gels a and b demonstrate, respectively, the relative positions of form III DNA, generated by Hpa II digestion (25), and of forms I and II, from the untreated sample. The form II band, found above the form I band, is very faint. After incubation of the untreated PM2 DNA in the $S_1$ buffer alone, a significant amount of the form I molecules were converted to form II (Fig. 6c). Incubation of 14.7 µg of PM2 DNA in the presence of 104 units of $S_1$ resulted in a reduction of the molecular weight of the DNA (Fig. 6d). Molecular weight markers, generated by Hind III digestion of PM2 DNA (17), gave several bands, the top 5 of which correspond to 5348, 4200, 2115, 1750 and 924 base pairs, respectively (Fig. 6e; M. T. Kuo, personal communication). The bands of 4200 and 1750 base pairs were due to mutant PM2 molecules in our sample which were not found in similar Hind III digestion of wild type PM2 DNA (25). Comparison of gels d and e demonstrated an approximate two- to five-fold reduction in molecular weight of the PM2.

Radiolabel analysis of the $S_1$ double-strand deoxyribonuclease activity was carried out using covalently closed circular polyoma DNA. Incubations performed under the same DNA/enzyme ratio as previously described demonstrated that only 2.9% of the native DNA was rendered TCA soluble.

Taken together, these results indicated that the molecular weight reduction of the PM2 DNA was not due to large amounts of double-strand exonuclease activity in the $S_1$ sample. Rather, it appears that a low level of endonuclease activity was present. Alternatively, it is possible that the nicking of one strand, introduced by the $S_1$ buffer alone, provided single-stranded regions which may be attacked by the $S_1$ and fragment the DNA molecule. Also, weakly hydrogen-bonded or strained regions in the closed circular DNAs examined, opened by the relatively high incubation temperature, may have been subject to $S_1$ digestion. In either case, it is apparent that these activities did not lead to a selective loss of sequences during the enzyme digestions.

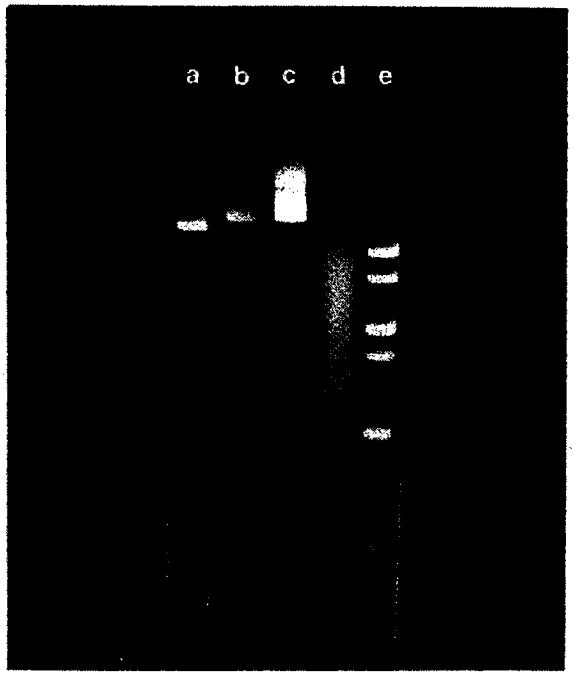


Figure 6. Double-strand deoxyribonuclease activity of $S_1$ nuclease. Native bacteriophage PM2 DNA was digested with either Hpa II (16) at 37°C for 2 hr, Hind III at 37°C for 24 hr in 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 40 mM NaCl or $S_1$ nuclease at 50°C for 10 min in $10^{-2}$ M NaCl, 3 x $10^{-2}$ M Na acetate, 3 x $10^{-5}$ M $ZnCl_2$, pH 4.5. The samples were electrophoresed through 1.4% agarose gels and the DNA was visualized by staining with ethidium bromide (16). a) linear, unit length form III PM2 DNA (0.5 µg) generated by Hpa II digestion, b) untreated PM2 DNA (1.0 µg) comprised mainly of form I with a small amount of form II, c) PM2 DNA (14.7 µg) incubated in $S_1$ buffer without enzyme, d) PM2 DNA (14.7 µg) incubated with 104 U $S_1$ nuclease, e) molecular weight markers of 5348, 4200, 2115, 1750 and 924 base pairs, (top 5 bands, top to bottom) generated by digestion of PM2 DNA (0.5 µg) with Hind III.

We also considered it necessary to assess the effect of SSC on the $S_1$ single-strand activity. The $S_1$ activity is increased by Zn cations (26), however, the citrate anion in the SSC was expected to chelate the $Zn^{++}$. As seen in Fig. 7, low concentrations of SSC apparently activated the enzyme and no inhibition occurred at concentrations below 0.5 X SSC (7.5 mM citrate). At 0.25 X SSC, the final concentration in the $S_1$ digestions after DNA reassociation, the $S_1$ activity was greater than 95% and the reaction essentially went to completion.

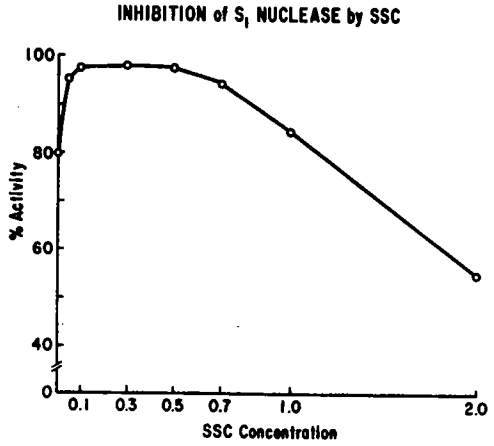


Figure 7. Inhibition of $S_1$ nuclease by SSC. A heat denatured mixture of [$^3$H]B. subtilis and unlabeled human placental DNAs (1:14) was incubated with 104 U $S_1$ nuclease at 50°C for 10 min in the presence of different concentrations of SSC. At 0.25 X SSC, the concentration used for digestion of Guinea pig DNA, the reaction went to completion.

## DISCUSSION

Three classes of highly repeated DNA sequences have previously been isolated from the Guinea pig genome. These sequences appear as satellites of the main band in $Ag^+Cs_2SO_4$ buoyant density gradients and together comprise 10.5% of the total DNA (1). By utilizing a procedure which is non-selective in the isolation of highly repeated sequences, we have obtained a fraction which constitutes 24% of the genome. The amount of snap-back DNA found in our sample, 2.7%, is in line with similar assays of three widely divergent DNAs. Analysis of Drosophila melanogaster (33), rat (34) and human (35) indicates that inverted repeats make up 3, 4 and 6% of these respective genomes. Allowing for these inverted repeat sequences, we find that the highly repeated sequences of the Guinea pig comprise 21% of the total DNA. Thus, about half of the highly repeated DNA has previously been masked by the main band of the buoyant density gradients. The high frequency of these sequences is compatible with the distribution of these same sequences in the C-band positive centromeric and short arm regions of Guinea pig chromosomes (10). The main band position, thermalyte distribution and fractionation in distamycin A-CsCl gradients possibly reflect the interspersion of these repeated DNAs, fairly high in AT content, with

non-repeated sequences. These adjacent unique DNAs would be removed during the $S_1$ nuclease digestion of the renatured duplexes. The occurrence of at least six distinct thermalytes indicates that there may be six or more different classes of highly repeated sequences in the Guinea pig genome. It may be possible to utilize thermal elution chromatography to obtain purified fractions of these reassociated DNAs for further studies.

The small amount of contaminating double-strand deoxyribonuclease activity present in the $S_1$ nuclease preparation may have an effect on the number of different density classes seen in the repetitive sample. A reduction in molecular weight of a molecule of DNA containing two adjacent classes of highly repeated sequences may yield two peaks of differing buoyant densities rather than one peak. Similarly, such cutting within one class of repetitive DNA may yield two DNAs of different density if the average AT content of the two halves of the sequence differ significantly. In order for this to occur, the action of the DNase would have to be non-random. Digestion of PM2 DNA in a non-random manner should yield at least one discrete band somewhere within the PM2 DNA digestion seen in Fig. 6d. Such a band is not seen in this digest. The site specific DNase activity cannot be ruled out, however, because the proper sites for the DNase attack may not be present within the PM2 DNA.

The double-strand DNase activity also does not appear to cause significant losses in the DNA. The average 2- to 5-fold reduction of molecular weight seen in the digestion of PM2 DNA (Fig. 6d) can most likely be attributed to nicking by the $S_1$ buffer alone (Fig. 6c) and the subsequent digestion of the single-strand region opposite the nick, rather than the double-strand activity. If the double-strand activity was responsible, the extent of the degradation of the PM2 DNA would not be expected to be so extensive. Although the nick and digest mechanism could also occur in the reassociated samples, it would not lead to a loss of DNA classes unless the nicking appeared only in particular sequences. Sequences which are weakly bonded or have a melting temperature below the 50°C used in the $S_1$ nuclease digestions may be expected to be particularly susceptible to such attack. Since the melting temperatures of the repeated sequences show a significant amount of DNA which denatures below 50°C we do not feel that the nicking is limited to the weakly bonded, early melting sequences.

It is noteworthy that our estimate of the amount of reassociated DNA sequences determined by spectrophotometry (Table II) is in excellent agreement with that derived by conventional radiochemical techniques (Table

I). The procedure of spectrophotometrically determining DNA concentrations in the presence of the enzyme may have general utility since the significant losses of DNA, commonly observed during deproteinizing procedures, can now be avoided.

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